

**This Page Is Inserted by IFW Operations
and is not a part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- **BLACK BORDERS**
- **TEXT CUT OFF AT TOP, BOTTOM OR SIDES**
- **FADED TEXT**
- **ILLEGIBLE TEXT**
- **SKEWED/SLANTED IMAGES**
- **COLORED PHOTOS**
- **BLACK OR VERY BLACK AND WHITE DARK PHOTOS**
- **GRAY SCALE DOCUMENTS**

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

AM

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/861, 5/10, A61K 48/00	A2	(11) International Publication Number: WO 00/39319 (43) International Publication Date: 6 July 2000 (06.07.00)
(21) International Application Number: PCT/US99/31249 (22) International Filing Date: 30 December 1999 (30.12.99) (30) Priority Data: 60/114,262 30 December 1998 (30.12.98) US 09/474,699 29 December 1999 (29.12.99) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/474,699 (CIP) Filed on 29 December 1999 (29.12.99) (71) Applicant (for all designated States except US): CALYDON, INC. [US/US]; 1324 Chesapeake Terrace, Sunnyvale, CA 94089 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HENDERSON, Daniel, R. [US/US]; 955 Matadero Avenue, Palo Alto, CA 94306 (US). YU, De-Chao [CN/US]; 1046 Eagle Lane, Foster City, CA 94404 (US). (74) Agents: POLIZZI, Catherine, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: TARGET CELL-SPECIFIC ADENOVIRAL VECTORS CONTAINING E3 AND METHODS OF USE THEREOF (57) Abstract The invention provides adenoviral vectors (preferably replication competent) comprising both an E3 sequence and at least one adenoviral gene under transcriptional control of a target cell-specific transcriptional response element. These vectors display significantly improved cytotoxicity, which is especially useful in the cancer context, in which selective destruction of target cells is desirable. The invention further provides host cells comprising the vectors. The invention further provides methods of using the adenoviral vectors.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TARGET CELL-SPECIFIC ADENOVIRAL VECTORS CONTAINING E3 AND METHODS OF USE THEREOF

5

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of U.S. Provisional Patent Application No.60/114,262, filed December 30, 1998. The priority application is hereby incorporated herein by reference in its entirety.

10

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

(Not applicable)

TECHNICAL FIELD

15

This invention relates to the field of adenoviral vectors and transfection. More specifically, the invention relates to target cell-specific adenoviral vectors containing E3.

BACKGROUND ART

20

In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by removing the mass surgically. If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to be eradicated.

25

30

A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells, while leaving unaffected the function of normal cells. For example, in prostate cancer therapy, the therapeutic ratio, or ratio of tumor cell killing to normal cell killing of traditional tumor chemotherapy, is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and prophylaxis of neoplasia are needed.

One possible treatment approach for many of these cancers is gene therapy, whereby a gene of interest is introduced into the malignant cell. Various viral vectors, including adenoviral vectors, have been developed as vehicles for gene therapy. The virtually exclusive focus in development of adenoviral vectors for gene therapy is use of adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus has been viewed as an undesirable result. In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity to the virus-infected cells. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression.

Adenovirus can cause persistent infections in humans and animals. The strategies of C type adenovirus (type Ad2 and Ad5) for evading host immune recognition are many, and generally involve E3, a delayed early transcription unit whose transcription is induced by the 289R E1A protein. During early stages of infection, the E3 promoter drives expression of nine alternatively spliced mRNAs that are polyadenylated at one of two sites, E3A and E3B. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199 (Pt.1):237-274. None of the E3 proteins is apparently required for adenovirus replication in cultured cells or in the lungs of hamsters or cotton rats, but they appear to play a role in evasion of host immune surveillance.

Six proteins which are encoded by the Ad-E3 region have been identified and characterized: (1) a 19-kDa glycoprotein (gp19k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k protein complex downregulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent

infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. Other studies have indicated that the E3-encoded 10.4K/14.5K complex proteins down-modulate the apoptosis receptor Fas/Apo-1. Elsing and Burger (1998) *Proc. Natl. Acad. Sci. USA* 95:10072-10077; and Shisler et al. (1997) *J. Virol.* 71:8299-8306. The functions of three E3-encoded proteins — 3.6k, 6.7k, and 12.5k — are currently unknown. Wold et al. (1995).

Traditionally, the pervasive dogma regarding the role of E3 in adenoviral vectors for gene therapy was that E3 should be deleted. E3 was viewed as non-essential for replication, and its deletion allowed insertion of foreign genes. Indeed, until quite recently, all adenoviral vectors lacked the E3 region.

More recently, it has been demonstrated that incorporation of E3 genes in the engineered adenovirus reduces the antiviral immune response and prolongs expression of foreign genes delivered by adenoviral vectors. It was shown that insertion of E3 genes in recombinant adenovirus facilitates re-administration of a functional vector for long-term gene expression and correction of an inherited metabolic disorder. Horwitz et al. (1995) *Curr. Topics Microbiol. Immunol.* 199(Pt 1):195-211. Other studies have indicated that, while expression from E3-deleted vectors is essentially turned off eight weeks after gene transfer, an E3-containing vector supported transgene expression with therapeutic levels of human factor IX *in vivo* for more than 4 months. Poller et al. (1996) *Gene Ther.* 3:521-530. The enhanced stability was attributed to efficient E3 region-mediated suppression of the host's antiviral immune response. More recently, it was demonstrated that a wild-type E3-containing adenoviral vector could direct prolonged expression of a non-immunogenic transgene. Persistence of this gene expression was also attributed to the presence of the E3 region. Wadsworth et al. (1997) *J. Virol.* 71:5189-5196. Further, when a recombinant adenovirus vector encoding hepatitis B surface antigen and containing an intact E3 region was used to infect chimpanzees, greater viral persistence, as indicated by the duration of virus shedding, was observed compared to counterpart vectors lacking E3. This phenomenon was attributed to evasion of host immune response. Chengalvala et al. (1997) *Vaccine* 15:335-339. However, the above-described E3-containing adenoviral vectors were

not replication competent and target cell specific. All of these studies employed adenovirus as a vehicle for expressing a transgene.

Use of adenoviral vectors as therapeutic vehicles for cancer has been reported. See, for example, Bischoff et al. (1996) *Science* 274:373-376; WO 96/349969; WO 96/17053.

5 Some of these approaches utilize target cell-type specific transcriptional regulatory elements to selectively drive adenoviral replication (and thus cytotoxicity). U.S. Pat. No. 5,698,443; see also WO 95/11984; WO 96/17053; WO 98/39465; WO 98/39467; WO 98/39466; and WO 98/39464. These vectors were deleted for E3.

10 Based on the teachings of the prior art, inclusion of E3 is not indicated in the context of using adenoviral vector replication for its cytotoxic effects (as opposed to using an adenoviral vectors as gene delivery vehicles), as suppression of the host's cytotoxic T cell response would not be considered a positive or desirable result. Further, inclusion of E3 into replication-competent adenoviral vectors would not be indicated since the well-accepted understanding in the art is that E3 is not necessary for viral replication.

15 Besides cancerous cells, it is often desirable to selectively destroy certain unwanted cells or tissues. Apart from surgery, however, which is invasive, there is a dearth of methods available, particularly non-invasive methods, which would allow such selective cytotoxicity and/or suppression.

20 There is a need for vector constructs that are capable of rapidly eliminating cancerous cells in a minimum number of administrations and which are suitable for use in cancer ablation treatments. There is also a need for an ability to selectively destroy, or impair, unwanted cells, regardless of cell type and/or regardless of anatomical location.

All publications and patent applications cited herein are hereby incorporated by reference in their entirety.

25 SUMMARY OF THE INVENTION

The invention provides target cell-specific adenoviral vectors (i.e., they are preferentially cytotoxic toward a specific target cell) comprising E3 (or an E3 sequence, or a portion of an E3 region), compositions, host cells, and kits comprising these vectors, and 30 methods using these vectors. Preferably, the vectors are replication competent. These

target cell-specific vectors express E3-encoded protein(s) and exhibit significantly greater cytotoxicity and/or enhanced adenoviral production per cell.

Accordingly, in one aspect, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and (b) an E3 region. In another aspect, an adenovirus vector of the invention comprises (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and (b) an E3 sequence. In some embodiments the adenoviral gene under transcriptional control of a target cell-specific TRE is one that is essential for adenoviral propagation, such as E1A and/or E1B. In some
10 embodiments, the target cell-specific TRE confers cell type-specific transcriptional regulation on the operably linked adenoviral gene. In other embodiments, the target cell-specific TRE is a cell status-specific TRE.

In other embodiments, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and (b) a portion of an E3 region.
15

In another aspect, the invention provides a replication competent adenovirus vector comprising an E3 sequence (or E3 region) under transcriptional control of a target cell specific TRE.

In another aspect, E3-containing, target-cell-specific adenoviral vectors of the invention further contain one or more transgenes. In some of these embodiments, a transgene can be under transcriptional control of a heterologous TRE, which may be a target cell-specific TRE.
20

In another aspect, the invention provides an adenoviral vector comprising an E3 region (or a portion of an E3 region, or an E3 sequence) under transcriptional control of a target-cell specific TRE.
25

In another aspect, the invention provides a host cell comprising an adenovirus vector(s) described herein.

In another aspect, the invention provides compositions comprising an adenovirus vector(s) described herein, preferably further comprising a pharmaceutically acceptable
30 excipient.

In another aspect, the invention provides kits which contain an adenoviral vector(s) described herein.

In another aspect, methods are provided for propagating an adenovirus specific for mammalian cells which permit the function of a target cell-specific TRE, said method comprising combining an adenovirus vector(s) described herein with mammalian cells that permit the function of a target cell-specific TRE, such that the adenovirus vector(s) enters the cell, whereby said adenovirus is propagated.

In another aspect, methods are provided for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, more particularly a target tumor cell, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

In another aspect, methods are provided for detecting a cell which allows the function of a target cell-specific TRE, which comprise contacting a cell in a biological sample with an adenovirus vector(s) of the invention, and detecting replication of the adenovirus vector(s), if any.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the E3 region of adenovirus. Letters above the boxes indicate known cellular locations of the corresponding polypeptide as follows: C, cytoplasm; ER M, endoplasmic reticulum membrane; NM, nuclear membrane; and PM, plasma membrane.

Figure 2 is a schematic representation of adenovirus vector CN702, which is wild-type but E3-deleted, and recombinant adenovirus vectors CN706 (lacking E3), CN739 (lacking E3), and CN787 (containing E3). With respect to adenoviral constructs (as opposed to precursor plasmid constructs), it is understood that "CN" and "CV" designations may be used interchangeably. For example, CN787 and CV787 refer to the same adenoviral construct.

Figure 3 is a bar graph depicting the titer, normalized to the titer of CN702 on 293 cells, of CN702 (first bars), CN706 (second bars), CN739 (third bars), and CN787 (fourth bars) in various cell lines.

Figure 4 is a half-tone reproduction of plaques formed by CN739 and CN787 on
5 293 cells.

Figure 5 is a graph depicting the extracellular viral yield, expressed as plaque-forming units (PFU), from LNCaP cells at various times post-infection, of adenoviral vectors CN739 (triangles) and CN787 (squares).

Figure 6 is a graph depicting, at various times post-infection, the total virus yield,
10 expressed as plaque-forming units (PFU), in LNCaP cells infected with CN739 (squares) or CN787 (diamonds).

Figure 7 is a graph depicting the results of an MTT assay for cytotoxicity, versus days post infection, of CN739-infected (circles), CN787-infected (squares), and mock-infected (triangles) LNCaP cells.

Figure 8 is a graph depicting viability, as measured by trypan blue exclusion, versus
15 days post infection of LNCaP cells infected with CN739 (squares) or CN787 (circles).

Figure 9 is a graph depicting relative tumor volumes over a period of five weeks in mice with tumor xenografts. The mice were treated with CN739 (squares), CN787 (circles), or vehicle alone (triangles).

Figure 10 is a graph depicting relative tumor volumes over a period of four weeks in mice with tumor xenografts. The mice were treated with 2.5×10^{11} PFU CN787 (squares), 3×10^{11} PFU CN787 (circles), or vehicle alone (triangles).

Figure 11 is a schematic representation of adenoviral vectors CN733 and CN790.

Figure 12 is a bar graph depicting the plaquing efficiency of CN702, CN733, CN739, and CN790 in various cell lines. For each cell line tested, the first, second, third and fourth bars represent the results obtained with CN702, CN733, CN739, and CN790, respectively. A star over a bar indicates no detectable plaques.

Figure 13 is a graph depicting the relative tumor volumes over a period of six weeks in mice with HepG2 tumor xenografts. Mice were treated with 10 injections of 1×10^{10} particles per dose of CN733 (diamonds), CN739 (squares), CN790 (triangles), or vehicle alone (crosses).
30

Figure 14 is a graph depicting relative serum concentrations of AFP in mice with tumor xenografts, after treatment with 10 injections of 1×10^{10} particles per dose of CN733 (diamonds), CN739 (squares), CN790 (triangles), or vehicle alone (crosses).

5 Figure 15 is a schematic representation of the 5' flanking region of the carcinoembryonic antigen (CEA) gene.

Figure 16 is a schematic representation of adenoviral constructs CN802, CN799, and CN798, all of which contain E3. Specificity and extent of killing are also indicated for each virus.

10 Figure 17 is a bar graph depicting the number of plaque-forming units 48 hours after infection of various cell lines with CN802 (left bars), CN799 (middle bars), or CN798 (right bars).

Figure 18 is a bar graph depicting the relative viral DNA yield obtained when various cell lines were infected with CN802 (stippled bars) or CN798 (solid bars). Stars indicate no detectable virus.

15 Figure 19 is a schematic representation of CN796 and CN797.

Figure 20 shows the nucleotide sequence of a hypoxia-responsive element (HRE) from the 5' flanking region of a rat enolase-1 gene (SEQ ID NO:7).

Figure 21 shows the nucleotide sequence of the 5' flanking region of a human E2F1 gene (SEQ ID NO:8). The asterisk indicates the transcription start site.

20 Figure 22 depicts a nucleotide sequence of a PSA-TRE (SEQ ID NO:9).

Figure 23 depicts a nucleotide sequence of a CEA-TRE (SEQ ID NO:10). The bent arrow above nucleotide 14,466 indicates the transcription start site.

Figure 24 depicts a nucleotide sequence of a human glandular kallikrein TRE (SEQ ID NO:11).

25 Figure 25 depicts a nucleotide sequence of a mucin TRE (SEQ ID NO:12).

Figure 26 depicts a nucleotide sequence of a rat probasin TRE (SEQ ID NO:13).

Figure 27 depicts the nucleotide sequence of the AFP-TRE driving expression of E1A in CN733 (SEQ ID NO:14).

30 Figure 28 depicts a nucleotide sequence of an AFP-TRE (SEQ ID NO:15).

Figure 29 is a half-tone reproduction of Southern blots of viruses hybridized with a 938 bp ³²P-labeled E1a probe (Figure 29A), or with an 881 bp ³²P-labeled E1b probe (Figure 29B).

Figure 30 is a graph depicting accumulation of deletion mutants, as measured by Molecular Imager scans of Southern blot radiograms as prepared in Figure 29B. CN790 was prepared serially through 5 passages in 293 cells at MOI = 10 (square) and MOI = 0.1 (diamond), and in HepG2 cells at MOI = 10 (closed circles) and MOI = 0.1 (triangles).

Figure 31 is a half-tone reproduction of plaques formed by CN733 and CN790 on 293 cells.

Figure 32 is a bar graph depicting the virus yield of CN790 (shaded bars) and wt Ad5 (open bars, CN802) in various cell lines.

Figure 33 is a half-tone reproduction of cells stained with crystal violet comparing cytotoxicity of CN790 and CN802 in various cell lines.

Figures 34A and 34B are bar graphs depicting the relative tumor volumes (Figure 34A) and relative serum AFP concentrations (Figure 34B) in nude mice bearing Hep3B tumor xenografts. In Figure 34A, mice were treated with 3 injections of 4 x 10⁹ pfu of CN790 (squares, n=7) or vehicle alone (triangles, n=8). Arrows indicate injection times. In Figure 34B, mice were treated with either CN790 (squares) or vehicle alone (triangles).

Figure 35 is a half-tone reproduction of immunohistochemical analysis of Hep3B xenograft tumors in nude mice treated with CN790. Adenovirus infected cells were detected by polyclonal rabbit antibody to Ad5 hexon and indicated by a filled arrow (Figure 35A). Apoptotic bodies were detected by TdT labeling and indicated by the filled arrow (Figure 35B). Photos at 400X.

MODES FOR CARRYING OUT THE INVENTION

We have discovered target cell-specific adenovirus vectors which contain an E3 region (or portion of an E3 region). In some embodiments, the adenovirus vectors of the invention contain an E3 sequence. Preferably, the adenovirus vector(s) is replication-competent, and, even more preferably, replicates preferentially in target cells. Inclusion of an E3 region (or E3 sequence, or portion of an E3 region) is contrary to the teachings of the prior art, which clearly indicated that deletion, not inclusion, of E3 would be desirable in

this context (i.e., conferring cell-specific cytotoxicity) due to E3's ability to dampen host cytotoxic response against infected cells. Moreover, the prior art teaching and emphasis that E3 is not necessary for viral replication indicates non-inclusion of E3 for replication-competent adenoviral vectors.

5 We have found that these vectors maintain their high level of specificity and are (a) significantly more cytotoxic; and/or (b) produce higher virus yield including extracellular virus yield; and/or (c) form larger plaques; and/or (d) produce rapid cell death; and/or (e) kill tumors or suppress tumor growth more efficiently in vivo than vectors lacking the E3 region. In addition, these vectors are target cell-specific, i.e., they comprise a target cell-
10 specific transcriptional regulatory element, such as a cell type- or cell status-specific transcriptional regulatory element(s) (TRE), that preferably drive an adenovirus gene essential for propagation, preferably one or more early genes. The vectors of the invention are useful for exerting selective cytotoxicity and/or suppressing cell growth (including tumor growth). This is especially useful in the cancer context, in which targeted cell killing
15 is desirable. This is also useful for targeted cytotoxic effects in other, non-tumor cells, when selective destruction and/or suppression of these cells is desirable. The vectors can also be useful for detecting the presence of cells which permit function of a target cell-specific TRE in, for example, an appropriate biological (such as clinical) sample. Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest
20 in a target cell by using a heterologous, i.e., non-adenoviral, TRE.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of
25 the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for
30 Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The

Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Definitions

An "E3 region" (used interchangeably with "intact E3 region") is a term well understood in the art and means the region of the adenoviral genome that encodes the E3 products (discussed herein). Generally, the E3 region is located between about 28583 and about 30470 of the adenoviral genome. An E3 region (or E3 sequence, defined below) for use in the present invention may be from any adenovirus serotype. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274.

An "E3 sequence" is a polynucleotide sequence that contains a sequence from an E3 region and excludes a sequence encoding only ADP. Thus, an E3 sequence can include ADP, as long as another portion of the E3 region is included. As is well known in the art, the ADP coding region is located in the E3 region within the adenoviral genome from about 29468 bp to about 29773 bp; including the Y leader, the location of ADP is from about 28375 bp to about 29773 bp for Ad5. Other ADP regions for other serotypes are known in the art. An E3 sequence includes, but is not limited to, deletions; insertions; fusions; and substitutions. An E3 sequence may also comprise an E3 region or a portion of the E3 region. It is understood that, as an "E3 sequence" is not limited to an "E3 region", alternative references herein to an "E3 region" or "E3 sequence" do not indicate that these terms are interchangeable. Assays for determining a functional E3 sequence for purposes of this invention are described herein.

An "E3 containing" vector of the invention refers to any of the embodiments described herein.

A "portion" of the E3 region means less than the entire E3 region, and as such includes polynucleotide deletions as well as polynucleotides encoding one or more polypeptide products of the E3 region. For purposes of this invention, when a "portion" of

E3 region includes a polynucleotide encoding ADP, that portion includes at least one other E3 polypeptide product, or a functional fragment of an E3 polypeptide product.

As used herein, a “transcriptional regulatory element”, or “TRE” is a polynucleotide sequence, preferably a DNA sequence, that regulates (i.e., controls) transcription of an operably-linked polynucleotide sequence by an RNA polymerase to form RNA. As used herein, a TRE increases transcription of an operably linked polynucleotide sequence in a host cell that allows the TRE to function. The TRE comprises an enhancer element and/or promoter element, which may or may not be derived from the same gene. The promoter and enhancer components of a TRE may be in any orientation and/or distance from the coding sequence of interest, and comprise multimers of the foregoing, as long as the desired transcriptional activity is obtained. As discussed herein, a TRE may or may not lack a silencer element.

As used herein, the term “target cell-specific TRE” is intended to mean that the TRE sequences to which a gene, which may be a gene essential for replication of an adenoviral vector, is operably linked, or to which a transgene is operably linked, functions specifically in that target cell so that transcription (and replication, if the operably linked gene is one essential for adenovirus replication) selectively or preferentially proceeds (i.e., when compared to a non-target cell) in that target cell, or so that a transgene polynucleotide is expressed in that target cell. This can occur by virtue of the presence in that target cell, and not in non-target cells (or significantly less so, or in inactive or less active forms, in non-target cells), of transcription factors that activate transcription driven by the operably linked transcriptional control sequences. It can also occur by virtue of the absence of transcription inhibiting factors that normally occur in non-target cells and prevent transcription driven by the operably linked transcriptional control sequences. “Target cell-specific TRE” includes cell type-specific and cell status-specific TRE, as well as “composite” TREs. The term “composite TRE” includes a TRE which comprises both a cell type-specific and a cell status-specific TRE. A target cell-specific TRE can also include a heterologous component, including, for example, an SV40 or a cytomegalovirus (CMV) promoter(s).

A “cell type-specific TRE” is preferentially functional, i.e., confers transcriptional activation, in a specific type of cell relative to other types of cells of different functionality.

“Cell type” is a reflection of a differentiation state of a cell which is, under normal physiological conditions, an irreversible, end-stage state. For example, a prostate-specific antigen TRE is functional in prostate cells, but is not substantially, or significantly, functional in other cell types such as hepatocytes, astrocytes, cardiocytes, lymphocytes, etc.

5 Generally, a cell type-specific TRE is active in only one cell type. When a cell type-specific TRE is active in more than one cell type, its activity is restricted to a limited number of cell types, i.e., it is not active in all cell types. A cell type-specific TRE may or may not be tumor cell specific. The term “cell type-specific”, as used herein, is intended to include cell type specificity, tissue specificity, as well as specificity for a cancerous state of

10 a given target cell type. In the latter case, specificity for a cancerous state of a normal cell is in comparison to a normal, non-cancerous counterpart.

As used herein, the term “cell status-specific TRE” is preferentially functional, i.e., confers transcriptional activation on an operably linked polynucleotide in a cell which allows a cell status-specific TRE to function, i.e., a cell which exhibits a particular

15 physiological condition, including, but not limited to, an aberrant physiological state. “Cell status” thus refers to a given, or particular, physiological state (or condition) of a cell, which is reversible and/or progressive. The physiological state may be generated internally or externally; for example, it may be a metabolic state (such as in response to conditions of low oxygen), or it may be generated due to heat or ionizing radiation. “Cell status” is

20 distinct from a “cell type”, which relates to a differentiation state of a cell, which under normal conditions is irreversible. Generally (but not necessarily), as discussed herein, a cell status is embodied in an aberrant physiological state, examples of which are given below.

A “normal cell status” or “normal physiological state” is the status of a cell which

25 exists in normal physiological conditions and which is non-dividing or divides in a regulated manner, i.e., a cell in a normal physiological state.

“Normal physiological conditions” are known to those skilled in the art. These conditions may vary, depending on a cell’s location in the body. For example, oxygen tension can vary from tissue to tissue. For *in vitro* analyses for the purposes of determining

30 whether a TRE is responsive to deviations from normal physiological conditions, these conditions generally include an oxygen concentration of about 20% O₂, and a temperature

of about 37°C. "Regulated cell division" is a term well understood in the art and refers to the normal mitotic activity of a cell. Those skilled in the art understand that normal mitotic activity varies from cell type to cell type. For example, many terminally differentiated cells in tissues exhibit little or no mitotic activity, while hematopoietic cells are generally mitotically active.

The terms "aberrant cell status" and "aberrant physiological state", used interchangeably herein, intend a condition of a cell which is a response to, a result of, or is influenced by, an aberrant physiological condition. An aberrant cell status is neither cell type-specific nor tissue type-specific. An aberrant cell status is defined in relation to a cell of the same type which is in a non-dividing/regulated dividing state and under normal physiological conditions. An "aberrant physiological condition" or "aberrant physiological state", as used herein, intends a condition which deviates from normal physiological conditions, and includes, but is not limited to, a physiological condition that is characterized by alterations in oxygen concentration, such as hypoxic conditions; temperatures which deviate from physiological temperatures; a condition that triggers apoptosis; radiation, including ionizing radiation and UV irradiation; de-regulated cell division, resulting for example, from a lack of, or insufficient amounts of, or inactivity of, a factor which controls cell division, such as, for example, retinoblastoma protein (Rb); variations in timing of cell cycle; infection with a pathogen; exposure to a chemical substance; or a combination of the above-listed conditions. Another example is a mutation that could, or does, exist in any cell type, i.e., its existence does not depend on, or is not related to, the differentiation state of the cell.

A "target cell", as used herein, is one that allows, permits or induces the function of a target cell-specific TRE such that it effects transcriptional activation (and/or enhancement), i.e., increases the level of transcription, of an operably linked polynucleotide. Target cell-specific TREs include cell type-specific TREs and cell status-specific TREs. Preferably, a target cell is a mammalian cell, preferably a human cell. A target cell may or may not be neoplastic.

"A target cell type which allows a TRE to function" or a cell in which the function of a TRE is "sufficiently preserved" or "functionally preserved", or "a cell in which a TRE is functional" is a cell in which the TRE, when operably linked to a promoter (if not

included in the TRE) and a reporter gene, increases expression of the reporter gene at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold, when compared to the expression of the same reporter gene when not operably linked to said TRE. In the context of cell type-specific TREs, a "target cell" is a cell type which allows the function of a cell type-specific TRE. Accordingly, in the context of a cell type-specific TRE, comparison is further made between expression of the reporter gene when operably linked to the cell type-specific TRE when in a target cell type versus when in a different cell type. In the context of cell status-specific TREs, a "target cell" is one which exhibits a given requisite physiological (or environmental) state, which may be an aberrant physiological state. Accordingly, in the context of a cell status-specific TRE, comparison is further made between expression of the reporter when operably linked to a cell status-specific TRE in the target cell in a given physiological state (which may be an aberrant physiological state) versus the same cell when in a normal physiological state (or a physiological state different from the given physiological state). Methods for measuring levels (whether relative or absolute) of expression are known in the art and are described herein.

A "functionally-preserved variant" of a target cell-specific TRE is a target cell-specific TRE which differs from another target cell-specific TRE, but still retains target cell-specific transcription activity, although the degree of activation may be altered (as discussed below). The difference in a target cell-specific TRE can be due to differences in linear sequence, arising from, for example, single base mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a target cell-specific TRE.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that expresses said gene. For example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which

increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor, such as a prostate cell. An *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “probasin (*PB*) transcriptional regulatory element”, or “*PB*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PB*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “prostate-specific antigen (*PSA*) transcriptional regulatory element”, or “*PSA*-TRE”, or “*PSE*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PSA*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PSA* promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “carcinoembryonic antigen (*CEA*) transcriptional regulatory element”, or “*CEA*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The *CEA*-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

As used herein, an “ α -fetoprotein (*AFP*) transcriptional regulatory element”, or “*AFP*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that

allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

5 As used herein, an “a mucin gene (*MUC*) transcriptional regulatory element”, or “*MUC1*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows a *MUC1*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to
10 transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

 An “adenovirus vector” or “adenoviral vector” (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in
15 an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically “mask” the molecule and/or increase half-life, and conjugated to a nonviral protein. Preferably, the polynucleotide is
20 DNA. As used herein, “DNA” includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

25 The terms “polynucleotide” and “nucleic acid”, used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or
30 derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted

sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH₂) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothiate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Mol. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software,

Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; and extend gap = 2. Another preferred program is the BLAST program for alignment of two nucleotide sequences, using default parameters as follows: open gap = 5; extension gap - 2 penalties; gap x dropoff = 50; expect = 10; word size = 11. The BLAST program is available at the following Internet address: <http://www.ncbi.nlm.nih.gov>.

A "replication competent" adenoviral vector or adenovirus is a term well-understood in the art and means that the adenoviral vector is able to replicate, or propagate. "Replication" and "propagation" are defined herein.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which a cell's usual biochemical or biological activities are compromised (i.e., inhibited). These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ³H-thymidine uptake, and plaque assays.

The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or induces a target cell-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a target cell-specific TRE to function (a non-target cell). Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker, such as prostate specific antigen.

In the context of adenovirus or adenovirus vector, a "heterologous polynucleotide" or "heterologous gene" or "transgene" (used interchangeably) is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be

expressed or present in the target cell prior to introduction by the adenovirus vector.

Examples of preferred transgenes are provided below.

In the context of adenovirus or adenovirus vector, a “heterologous” promoter or enhancer or TRE is one which is not associated with or derived from an adenovirus gene.

5 In the context of adenovirus or adenovirus vector, a “heterologous target cell-specific TRE” is a target cell-specific TRE which is not associated with or derived from an adenovirus gene.

In the context of adenovirus or adenovirus vector, an “endogenous” promoter, enhancer, or TRE is native to or derived from adenovirus.

10 In the context of a target cell-specific TRE, a “heterologous” promoter or enhancer is one which is not normally associated in a cell with or derived from the target cell-specific TRE. Examples of a heterologous promoter or enhancer are the albumin promoter or enhancer and other viral promoters and enhancers, such as SV40 or CMV, or cell type specific TREs such as a prostate specific TRE (for example, a prostate specific TRE is
15 heterologous with respect to a liver cell specific TRE).

“Suppressing” tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are
20 proliferating using a ³H-thymidine incorporation assay, or counting tumor cells. “Suppressing” tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms “neoplastic cells”, “neoplasia”, “tumor”, “tumor cells”, “cancer” and “cancer cells”, (used interchangeably) refer to cells which exhibit relatively
25 autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

A “host cell” includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single
30 host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate

mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

“Replication” and “propagation” are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay, plaque assay, or a one-step growth curve. “Replication” and “propagation” include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

“Replicating preferentially” means that the adenovirus replicates more in a target cell than in a non-target cell. Preferably, the adenovirus replicates at least about 10%, more preferably at least about 25%, more preferably at least about 50%, more preferably at least about 100% (i.e., 2-fold) higher, preferably at least about 5-fold higher, more preferably at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400-fold to about 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about 1×10^6 higher.

A polynucleotide sequence that is “depicted in” a SEQ ID NO means that the sequence is present as, or contained within an identical contiguous sequence in the SEQ ID NO. The term encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

A “biological sample” encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term “biological sample”

encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and pets.

An "effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. An effective amount can be administered in one or more administrations. In the context of a disease state, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

E3-containing target cell-specific adenoviral vectors

The invention provides target cell-specific adenovirus vectors comprising an E3 sequence and an adenoviral gene under transcriptional control of a target cell-specific TRE. Preferably, the vectors are replication-competent. Even more preferably, the adenoviral gene under transcriptional control of a target cell-specific TRE is one essential for adenoviral replication. Inclusion of the E3 region of adenovirus was found to enhance cytotoxicity of the target cell-specific adenoviral vectors of the present invention.

E3-containing adenoviral vectors of the invention (including adenoviral vectors containing E3 sequences and/or a portion of E3) maintain their high level of specificity and display one or more of the following characteristics relative to adenoviral vectors lacking an E3 region (or portion of E3), i.e., they (a) are more cytotoxic; (b) produce higher virus yield, including intracellular and extracellular virus yield; (c) form larger plaques; (d) produce rapid cell death; (e) kill tumors more efficiently *in vivo*; and/or (f) suppress tumor growth more efficiently *in vivo*. Any detectable increase is sufficient, although preferably any of these characteristics may be increased (when compared to adenoviral vectors lacking E3) at least about 10%, more preferably at least about 25%, more preferably at least about 50%, more preferably at least about 100% (i.e., 2-fold), more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 20-fold or more. In some embodiments, cytotoxicity of an E3-containing adenoviral vector of the invention is increased when compared to the cytotoxicity of an adenoviral counterpart lacking E3. It is understood that an E3-containing adenoviral vector of the invention can exhibit the

aforementioned increased level(s) of activity with respect to one or more of these characteristics, to the same or different degree relative to an adenoviral counterpart lacking E3. For example, a given E3-containing adenoviral vector may exhibit 2-fold greater cytotoxicity, yet produce a 3-fold higher virus yield than an adenoviral counterpart lacking E3.

Cytotoxicity can be measured by any known method, including, but not limited to, an MTT assay and trypan blue dye exclusion, both of which are described in Example 2. Virus yield can be measured by any known method, including, but not limited to, plaque assay on a cell line permissive for adenoviral replication, as described in Example 2.

Whether a given E3-containing adenovirus vector forms larger plaques than a counterpart vector lacking E3 can be determined, for example, by plaque assay and measuring plaque diameter. Whether a given E3-containing adenovirus vector produces rapid cell death relative to a counterpart adenovirus vector lacking E3 can be determined using any known method, including, but not limited to, MTT assay or trypan blue exclusion assay performed over a suitable course of time post-infection, such as 1, 2, 3, 5, or 7 or more days post-infection. Tumor killing and suppression of tumor cell growth can be measured by any known method, including, but not limited to, a tumor xenograft assay as described in Example 2.

It is understood that, for purposes of this invention, not only may an (intact) E3 region or a portion of an E3 region be used, but E3 sequences (which include other forms and arrangements of E3) may be employed, including, but not limited to, deletions; internal deletions; fusions comprising E3 or a portion of E3; insertions (whereby one or more nucleotides are inserted within an E3 sequence); and substitutions, including silent substitutions, as well as substitutions resulting in one or more differences in an amino acid sequence(s). A functional E3 sequence may be determined by assaying, for example, for any of the characteristics listed above.

The adenoviral vectors of this invention contain an E3 sequence, which may be an intact E3 region or a portion of an E3 region. It is understood that, as inclusion of E3 confers observable and measurable functionality on the adenoviral vectors, for example, increased replication and production, functionally equivalent (in which functionality is essentially maintained, preserved, or even enhanced or diminished) variants of E3 may be

constructed. Functional equivalents of an E3 region include those in which one or more E3 functions is essentially maintained, preserved, or even enhanced or diminished which confer one or more of the following properties on the virus: (a) cytotoxicity; (b) production of virus yield, including intracellular and extracellular virus yield; (c) plaque formation; and (d) production of cell death; (e) killing and/or suppressing of tumors *in vivo*. For example, portions of E3 may be used. As is explained in the definition of "portion" of E3, a portion may be, non-inclusively, either of the following: (a) deletion, preferably at the 3' end; (b) inclusion of one or more various open reading frames of E3. Five proteins which are encoded by the Ad-E3 region have been identified and characterized: (1) a 19-kDa glycoprotein (gp19k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k protein complex downregulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. The functions of three E3-encoded proteins -- 3.6k, 6.7k and 12.5k -- are unknown. A ninth protein having a molecular weight of 7.5 kDa has been postulated to exist, but has not been detected in cells infected with wild-type adenovirus. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. The E3 region is schematically depicted in Figure 1. These intact, portions, or variants of E3 may be readily constructed using standard knowledge and techniques in the art. Preferably, an intact E3 region is used. Appropriate vectors comprising an intact E3 region are commercially available, e.g., from Microbix. In other embodiments, an E3 region is used which lacks the 10.4k/14.5k complex and the 14.7 protein (i.e., deletion of about bp 29781 to about bp 30847). E3 sequences or portions of E3 can be derived from such vectors using standard techniques of molecular biology, such as restriction and ligation, and polymerase chain reactions. Any of the various serotypes of adenovirus can be used in the present invention, such as Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, serotype Ad5 is exemplified herein.

In the adenovirus vectors of the present invention, E3 (i.e., E3 sequence, which includes E3 region or portion of an E3 region) may or may not be under transcriptional control of native adenoviral transcriptional control element(s). The E3 promoter is located within the coding sequence for virion protein VIII, an essential protein which is highly conserved among adenovirus serotypes. In some embodiments, E3 is under transcriptional control of a heterologous TRE, including, but not limited to, a target cell-specific TRE. In one embodiment, the invention provides an adenoviral vector, preferably replication competent, that comprises an E3 sequence under transcriptional control of a target-cell specific TRE. A number of target cell-specific TREs are known in the art, several of which are described in more detail herein. In other embodiments, the E3 region is under transcriptional control of a target cell-specific TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE.

Target cell-specific TREs

Adenoviral vectors of the present invention include, in addition to an E3 sequence, one or more adenoviral genes under transcriptional control of a target cell-specific TRE, and optionally include a transgene, which may also be under transcriptional control of a target cell-specific TRE or other TRE. Preferably, the adenovirus gene(s) under transcriptional control of a target cell-specific TRE is essential for propagation, preferably an early gene(s), such as E1A, E1B, and/or E4. When the adenovirus vector(s) is selectively (i.e., preferentially) replication competent for propagation in target cells, these cells will be preferentially killed upon adenoviral proliferation. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic replication, the adenovirus vector replication is significantly reduced, thus lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures may be retained to monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for occurrence (i.e., presence) and/or recurrence of the target cell, e.g., a neoplastic cell or other undesired cell. To further ensure cytotoxicity, one or more transgenes having a cytotoxic effect may also be present and under selective transcriptional control. In this embodiment, one may

provide higher confidence that the target cells will be destroyed. Target cell-specific TREs include cell type-specific TREs and cell status-specific TREs, either of which may be specific for a neoplastic cell. Alternatively, an adenovirus gene, and optionally, a transgene, can be under transcriptional control of a cell type-specific and a cell status-specific TRE. Such target cell-specific TREs are termed "composite" target cell-specific TREs herein.

Target cell-specific TREs for use in the adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been described which are expressed preferentially in specific cell types. Any of these cell type-specific genes can be used to generate a cell type-specific TRE. Similarly, a number of genes have been described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

A target cell-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., cell in a normal cell state). Thus, presence of a silencer may confer enhanced target cell-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced target cell-specific replication due to more effective replication in target cells.

A target cell-specific TRE can comprise multimers, for example, a tandem series of at least two, at least three, at least four, or at least five target cell-specific response elements. These multimers may also contain heterologous promoter and/or enhancer sequences.

In some embodiments, an E3-containing adenoviral vector of the invention further comprises a first adenoviral gene under transcriptional control of a first target cell-specific TRE and a second gene under transcriptional control of a second target cell-specific TRE. In some of these embodiments, the first adenoviral gene is one essential for adenoviral replication. In other embodiments, the first and the second genes are adenoviral genes. In other embodiments, the first and the second genes are adenoviral genes essential for replication.

The first and the second target cell-specific TREs may or may not be substantially identical to one another. By "substantially identical" is meant a requisite degree of sequence identity between the two TREs. The degree of sequence identity between these TREs is at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and most preferably 100%.

Adenoviral constructs in which the first and second target cell-specific TREs are substantially identical, particularly if these TREs control transcription of early genes (such as E1A and E1B), may display an instability which may be desirable in certain contexts, such as when an automatic "self-destruction" property can shut down the virus, thereby controlling the degree of propagation. Conversely, more stable double (or more) constructs may be desirable in yet other contexts. In either case, we have observed that having multiple adenovirus genes essential for propagation (especially early genes, more particularly E1a and E1b), confers significant specificity to the vectors. In some embodiments, the first and second target cell-specific TREs are cell type-specific and are functional in the same cell type.

Sequence identity can be determined by a sequence comparison using, i.e., sequence alignment programs that are known in the art, such as those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; and extend gap = 2. Another preferred program is the BLAST program for alignment of two nucleotide sequences, using default parameters as follows: open gap = 5; extension gap - 2 penalties; gap x dropoff = 50; expect = 10; word size = 11. The BLAST program is available at the following Internet address: <http://www.ncbi.nlm.nih.gov>. Alternatively, hybridization under stringent conditions can also indicate degree of sequence identity.

Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction of widely known and published in the art. See, for example, Sambrook et al. (1989). Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of

25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X SSC, or deionized water.

"T_m" is the temperature in degrees Celsius at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in anti-parallel direction by Watson-Crick base pairing dissociates into single strands under conditions of the experiment. T_m may be predicted according to a standard formula, such as:

$$T_m = 81.5 + 16.6 \log[X^+] + 0.41 (\%G/C) - 0.61 (\%F) - 600/L$$

where [X⁺] is the cation concentration (usually sodium ion, Na⁺) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

Stringent conditions for both DNA/DNA and DNA/RNA hybridization are as described by Sambrook et al. *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, herein incorporated by reference. For example, see page 7.52 of Sambrook et al. An example of stringent hybridization conditions are 68°C, 0.2 x SSC.

Accordingly, in some embodiments, the invention provides an adenoviral vector comprising, in addition to an E3 sequence, any of the following: (a) E1A under transcriptional control of a *PB*-TRE and E1B under transcriptional control of a *PSA*-TRE; (b) E1A under transcriptional control of an *AFP*-TRE and E1B under transcriptional control of a second (but not necessarily identical or non-identical) *AFP*-TRE; (c) E1A under transcriptional control of a *CEA*-TRE. As the discussion of possible TREs below makes clear, these are non-limiting examples of the possible combinations for the E3-containing vectors of the invention.

An example of an adenoviral vector comprising, in addition to an E3 sequence, E1A under transcriptional control of a *PB*-TRE and E1B under transcriptional control of a *PSA*-TRE is the construct CN787, the construction of which is described in detail in Example 1.

An example of an adenoviral vector comprising, in addition to an E3 sequence, E1A under transcriptional control of an *AFP*-TRE and E1B under transcriptional control of a second (but not necessarily identical or non-identical) *AFP*-TRE is the construct CN790, which is described in Example 1. Examples of an adenoviral vector comprising, in addition to an E3 sequence, E1A under transcriptional control of a *CEA*-TRE are CN798 and CN799, which are described in detail in Example 1. Accordingly, the present invention encompasses, as specific embodiments, CN787, CN790, CN798, and CN799 (alternatively and interchangeably denoted CV787, CV790, CV798, and CV799).

In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first target cell-specific TRE, and a transgene under control of a second target cell-specific TRE. The first and the second target cell-specific TREs may or may not be substantially identical to one another.

In some embodiments, a target cell-specific TRE can comprise two or more different types of TREs. For example, a cell status-specific TRE can be juxtaposed with another TRE, such as a different cell status-specific TRE, or, alternatively, a cell type-specific TRE. "Juxtaposed" means a cell status-specific TRE and the second TRE transcriptionally control the same gene. For these embodiments, the cell status-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene. For example, as described in Example 1 and shown in Figure 19, a cell type-specific TRE can be juxtaposed with a cell status-specific TRE to control transcription of an operably linked adenoviral gene. The construct CN797 is an example of an E3-containing adenoviral vector comprising the composite TRE described in Example 1. Accordingly, the present invention encompasses, as one embodiment, CN797 (alternatively and interchangeably denoted CV797). Such "composite" TREs can be used to confer both cell status- and cell type-specific expression of an operably linked polynucleotide, and thus may confer significantly greater specificity and/or efficacy. Examples of cell type-specific TREs are provided below. Alternatively, "composite" TREs can be used to confer different, and possibly synergistic, cell status and/or cell type specificity. For example, a composite cell status-specific TRE could

confer specificity to hypoxia and heat shock. Example 1 provides a description of an E3-containing adenovirus construct comprising E1A and E1B genes under transcriptional control of a combination transcriptional regulatory element consisting of a hypoxia-responsive element (HRE) and a prostate-specific TRE, PSA-TRE. Accordingly, in one
5 embodiment, an HRE comprises a 67-base fragment depicted in Figure 20 (SEQ ID NO:7).

As is readily appreciated by one skilled in the art, a target cell-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene
10 assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite target cell-specific transcription function. Hence, the invention also includes functionally-preserved variants of the nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications
15 will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

The activity of a TRE generally depends upon the presence of transcriptional regulatory factors and/or the absence of transcriptional regulatory inhibitors. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operatively
20 linked to) the TRE. As discussed herein, a TRE can be of varying lengths, and of varying sequence composition.

Certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and gene activation. One of skill in the art would recognize that some alterations of bases in and around known the transcription factor binding sites are
25 more likely to negatively affect gene activation and cell-specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such

effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in TRE functional and TRE non-functional cells. Additionally, one of skill in the art would
5 recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

As an example of how target cell-specific TRE activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant
10 methods. The sequence(s) to be tested is inserted into a vector containing an appropriate reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT), β -galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), green fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus
15 constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative target cell-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE-dextran. Suitable host cells include any cell type, including but not limited to, Hep3B, Hep G2, HuH7, HuH1/C12, LNCaP, HBL-100, Chang liver cells,
20 MCF-7, HLF, HLE, 3T3, HUVEC, and HeLa. For a TRE suspected to be a cell status-specific TRE, host cells transfected with the TRE-reporter gene construct to be tested are subjected to conditions which result in a change in cell status (for example, one which result in an aberrant physiological state). The same cells not subjected to these conditions, i.e., which are under normal physiological conditions and therefore in a normal
25 physiological state, serve as controls. Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between cells in a particular state and control indicates presence or absence of transcriptional activation. Generally, to determine cell specific activity of a TRE, the TRE-reporter gene constructs are introduced into a variety of cell types. The amount of TRE activity is
30 determined in each cell type and compared to that of a reporter gene construct without the

TRE. A TRE is cell specific when it is preferentially functional in a specific type of cell over a different type of cell. "Transcriptional activation" has been defined above.

Comparisons between or among various target cell-specific TREs can be assessed, for example, by measuring and comparing levels of expression within a single cell line under normal and aberrant physiological conditions (for cell status-specific TREs) or within a single cell line of the appropriate cell type (for cell type-specific TREs). These comparisons may also be made by measuring and comparing levels of expression within a single cell line. It is understood that absolute transcriptional activity of a target cell-specific TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the target cell-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the cytomegalovirus (CMV) immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

As an example of how hypoxia induction can be measured, one can use an assay such as that described in Jiang et al. (1997) *Cancer Research* 57:5328-5335 or Dachs et al. (1997) *Nature Med.* 3:515-520. For example, a construct comprising a putative HRE, or multiple tandem copies thereof, together with a minimal promoter element, operably linked and controlling transcription of a polynucleotide which encodes a protein which is detectable or can be used to give a detectable signal, is introduced into host cells. The host cells are then subjected to conditions of normoxia (e.g., 20% O₂), and varying degrees of hypoxia, such as 5%, 2%, 1%, 0.1%, or less, O₂. The expression product of the operably linked polynucleotide (reporter gene) is then measured.

As an example of how a prostate cell-specific TRE activity, such as a PSA-TRE, can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing an appropriate reporter gene encoding a reporter protein, as described

above. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative PSA-TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran. Suitable host cells include any cell type that produces androgen receptor, including but not limited to, prostate cells, including prostate tumor cells such as LNCaP. A gene encoding androgen receptor can be transformed into and expressed in any cell that does not normally express AR; in such a cell, a PSA-TRE will be functional. Non-androgen receptor producing cells, such as HLF, HLE, and 3T3 and the non-AR-producing prostate cancer cells PC3 and DU145 can be used as a control. Results are obtained by measuring the level of expression of the reporter gene using standard assays. The comparison of reporter gene expression between AR-producing cells and the control cell indicates the presence, absence, and/or degree of transcriptional activation.

Alternatively a putative cell status-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells exhibiting the requisite physiological state, such as heat or ionizing radiation. For this assay, constructs containing an adenovirus gene essential to replication operably linked to a putative cell status-specific TRE are transfected into cells which exhibit the requisite physiological state. Viral replication in those cells is compared, for example, to viral replication by the construct in cells under normal physiological conditions (i.e., not exhibiting the requisite physiological state).

When a target cell-specific TRE is used with an adenovirus gene that is essential for propagation replication, competence is preferentially achievable in the target cell type and/or the target cell exhibiting a particular cell status.

Cell type-specific TREs

In some embodiments, the E3-containing adenoviral vectors of the invention comprise a cell type-specific TRE, such as a prostate cell-specific TRE, a liver cell-specific TRE, a breast cancer cell-specific TRE, or a colon cancer cell-specific TRE. A cell type-specific TRE is preferentially functional in a specific type of cell relative to other types of cells of different functionality. For example, TREs that function preferentially in prostate cells (i.e., are prostate cell-specific) include, but are not limited to, TREs derived from the

prostate-specific antigen gene (*PSA-TRE*) (U.S. Patent No. 5,648,478), the glandular kallikrein-1 gene (from the human gene, *hKLK2-TRE*), and the probasin gene (*PB-TRE*) (International Patent Application No. PCT/US98/04132). All three of these genes are preferentially expressed in prostate cells and the expression is androgen-inducible.

5 Generally, expression of genes responsive to androgen induction requires the presence of an androgen receptor (AR).

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a *PSA-TRE*. *PSA* is synthesized exclusively by normal, hyperplastic, and malignant prostatic epithelia; hence, its tissue-specific expression has made it an excellent
10 biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal serum levels of *PSA* are typically below 5 ng/ml, with elevated levels indicative of BPH or CaP. Lundwall et al. (1987) *FEBS Lett.* 214: 317; Lundwall (1989) *Biochem. Biophys. Res. Comm.* 161: 1151; and Riegmann et al. (1991) *Molec. Endocrin.* 5: 1921.

The region of the *PSA* gene that is used to provide cell specificity dependent upon
15 androgens, particular in prostate cells, involves approximately 6.0 kilobases. Schuur et al. (1996) *J. Biol. Chem.* 271:7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the *PSA* gene. The *PSA* promoter consists of the sequence from about nt -540 to nt +8 relative to the transcription start site. Juxtapositioning of these two genetic elements yield
20 a fully functional, minimal prostate-specific enhancer/promoter (*PSA-TRE*). Other portions of the approximately 6.0 kb region of the *PSA* gene can be used in the present invention, as long as requisite functionality is maintained. Accordingly, in some embodiments, a *PSA-TRE* comprises nucleotides -5322 to -3739 juxtaposed to nucleotides -540 to +8 relative to the transcription start site of the *PSA* gene.

25 In Example 1, adenoviral vector CN797 is described which comprises a composite TRE comprising an HRE and a *PSA-TRE*, the *PSA-TRE* comprising a *PSA* enhancer from -5322 to -3738 fused to a *PSA* promoter from -541 to +12. This *PSA-TRE* is derived from adenoviral vector CN706. Rodriguez et al. (1997) *Cancer Research* 57:2559-2563. Accordingly, in one embodiment an adenoviral vector comprises an adenovirus E1A gene
30 under transcriptional control of a composite TRE comprising the cell status-specific TRE, HRE, and a cell type-specific TRE, a *PSA-TRE*.

The *PSE* and *PSA* TRE used in the present invention are derived from sequences depicted in and Figure 22 (SEQ ID NO:9).

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a probasin TRE. The rat probasin (PB) gene encodes a nuclear and secreted protein, probasin, that is only expressed in the dorsolateral prostate. Dodd et al. (1983) *J. Biol. Chem.* 258:10731-10737; Matusik et al. (1986) *Biochem. Cell. Biol.* 64: 601-607; and Sweetland et al. (1988) *Mol. Cell. Biochem.* 84: 3-15.

The PB-TREs used in this invention are derived from mammalian cells, including but not limited to, rodent cells, such as rat. In the Examples provided herein, a PB-TRE is derived from rat cells. In one embodiment, the PB-TRE comprises a promoter of a probasin gene. In one embodiment, the PB-TRE comprises an enhancer from a probasin gene. In another embodiment, the PB-TRE comprises a promoter from a probasin gene and an enhancer from a probasin gene. In certain embodiments wherein the PB-TRE comprises an enhancer from a probasin gene, the enhancer may be in combination with a promoter from a probasin gene or a promoter from another gene. In certain embodiments wherein the PB-TRE comprises a promoter from a probasin gene, the promoter may be in combination with an enhancer from a probasin gene or an enhancer from another gene. In addition, the PB-TRE can comprise multiple promoters and/or multiple enhancers derived from the probasin gene or another gene or other genes.

A DNA fragment comprising the 5'-flanking PB DNA, nt about -426 to about +28 (SEQ ID NO:13), carries sufficient information to direct prostate-specific, developmentally- and hormonally-regulated expression of a heterologous (non-probasin) gene in transgenic mice. Greenberg et al. (1994) *Mol. Endocrinol.* 8: 230-239; Foster et al. (1997) *Cancer Res.* 57:3325-30. Furthermore, this expression was both male-specific and restricted to the epithelial cells of the lateral, dorsal, and ventral prostate lobes. The demonstration that the foreign gene activity approached precastration levels when transgenic mice were supplemented with testosterone indicates that the PB-driven reporter transgene was responding to androgens *in vivo*. Moreover, a PB-TRE could drive expression of the simian virus 40 large tumor antigen-coding region in the prostate of the transgenic mice. Greenberg et al. (1995) *Proc. Natl. Acad. Sci.* 92: 3439-3443.

Accordingly, in one embodiment, a PB-TRE is the sequence upstream of the probasin coding segment, comprising, for example, the sequence given in SEQ ID NO:13. This sequence, e.g. from about -426 to about +28 relative to the transcriptional start site, comprises protein binding sites believed to be important or essential in cell-specific transcription, including ARE-1, ARE-2, a CAAT box, and a TATAA box.

Alternatively, a PB-TRE comprises, for example, the fragment of DNA upstream of the PB gene between base pairs about -286 and about +28 relative to the transcriptional start (nucleotides about 141 to about 454 of SEQ ID NO:13). Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36. Sequence analysis revealed that this PB-TRE contains two ARE sites (designated ARE-1, also known as ARBS-1, which resembles a glucocorticoid response element, at about -236 to about -223 relative to the transcriptional start (nucleotides about 191 to about 204 of SEQ ID NO:13); and ARE-2, also known as ARBS-2, which is a unique sequence at about -140 to about -117 (nucleotides about 286 to about 310 of SEQ ID NO:13) required for androgen regulation. A single base mutation in ARE-1 or ARE-2 can result in the loss of androgen induction. Rennie et al. (1993) *Mol. Endocrinol.* 7:23-36. A fragment of 5'-flanking PB DNA containing the two ARE sites could drive expression of the bacterial chloramphenicol acetyltransferase (CAT); expression was prostate-specific and inducible by androgens, but not by glucocorticoids. Greenberg et al. (1994) *Mol. Endocrinol.* 8:230-239. Like the probasin gene, the AR gene itself is regulated by two ARE sites upstream of the coding segment. The first AR gene ARE site, ARE-1, resembles a half-site of the palindromic hormone response element and the second, ARE-2, is identical to a portion of the probasin sequence. Dai et al. (1996) *Mol. Endocrinol.* 10:1582-94. A PB enhancer is exemplified by an ARE site or pair of ARE sites, or any other sequence capable of assisting a promoter in prostate-specific transcription. Proper spacing between ARE sites may also be important in their function.

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a human glandular kallikrein-2 TRE (*hKLK2*-TRE). The *hKLK2* gene encodes human glandular kallikrein-2 (hK2), a protein which is synthesized virtually exclusively in prostate cells and whose synthesis is upregulated by androgens, primarily by transcriptional activation of *hKLK2*. Wolf et al. (1992) *Molec. Endocrinol.* 6:753-762; Morris (1989)

Clin. Exp. Pharm. Physiol. 16:345-351; Qui et al. (1990) *J. Urol.* 144:1550-1556; Young et al. (1992) *Biochem.* 31:818-824.

hKLK2 promoter activity has been described. Schedlich et al. (1987) *DNA* 6:429-437; and Murtha et al. (1993) *Biochem.* 32:6459-6464. Promoter activity has been attributed to nucleotides about 11,290 to about 12,047 of SEQ ID NO:11. Accordingly, in some embodiments, an *hKLK2* TRE comprises an *hKLK2* promoter comprising nucleotides about 11,290 to about 12,047 of SEQ ID NO:11.

hKLK2 enhancer activity is found within nucleotides 1 through 9765 of SEQ ID NO:11 (corresponding to -12,014 to -2257 relative to the transcription start site). Portions of this region have been identified which retain enhancer function. Enhancer activity has been demonstrated in the region from nucleotides 8021 to 8371 of SEQ ID NO:11 (corresponding to -3993 to -3643 relative to the transcription start site), as described in PCT/US98/16312. Accordingly, the invention includes an isolated polynucleotide sequence comprising nucleotides about 8021 to about 8371 of SEQ ID NO:11. Enhancer activity has also been demonstrated in the region from nucleotides 7200 to 8371 of SEQ ID NO:11 (corresponding to -4814 to -3643 relative to the transcription start site), as described. Accordingly, the invention includes an isolated polynucleotide sequence comprising about 7200 to about 8371 of SEQ ID NO:11. Enhancer activity has further been demonstrated in the region from 6859 to 8627 of SEQ ID NO:11 (-5155 to -3387 relative to the transcription start site). Accordingly, the invention includes an isolated polynucleotide sequence comprising about 6859 to about 8627 of SEQ ID NO:11. Enhancer activity has been demonstrated in the region from 5976 to 9620 of SEQ ID NO:11 (-6038 to -2394 relative to the transcription start site). Accordingly, the invention includes an isolated polynucleotide sequence comprising about 5976 to about 9620 of SEQ ID NO:11. An active enhancer lies within an XhoI-ApaI fragment spanning a region from about 2 to about 6 kb upstream of the *hKLK2* structural gene. Accordingly, the invention further includes an isolated polynucleotide comprising nucleotides about 1 through about 9765 of SEQ ID NO:11. For each of these embodiments, the polynucleotide has enhancer activity.

In the present invention, cell type-specific TREs may be preferentially functional in particular tumor cells. Non-limiting examples of tumor cell-specific TREs, and non-

limiting examples of respective potential target cells, include TREs from the following genes: α -fetoprotein (*AFP*) (liver cancer), mucin-like glycoprotein DF3 (*MUC1*) (breast carcinoma), carcinoembryonic antigen (*CEA*) (colorectal, gastric, pancreatic, breast, and lung cancers), plasminogen activator urokinase (*uPA*) and its receptor gene (breast, colon, and liver cancers), *HER-2/neu* (*c-erbB2/neu*) (breast, ovarian, stomach, and lung cancers).

Other cell type-specific TREs may be derived from the following exemplary genes (cell type in which the TREs are specifically functional are in parentheses): vascular endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver), von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in smooth muscle), synthetase I (small intestine), Na-K-Cl transporter (kidney). Additional cell type-specific TREs are known in the art, and some exemplary TREs are discussed below.

In some embodiments, the E3-containing adenovirus vectors of the invention comprise an AFP-TRE. AFP is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with advanced disease. The serum AFP levels in patients appear to be regulated by AFP expression in hepatocellular carcinoma but not in surrounding normal liver. Thus, the AFP gene appears to be regulated to hepatoma cell-specific expression.

Cell type-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe et al. (1987) *J. Biol. Chem.* 262:4812-4818. The entire 5' *AFP* flanking region (containing the promoter, putative silencer, and enhancer elements) is contained within approximately 5 kb upstream from the transcription start site.

The *AFP* enhancer region in human is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP* gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements yields a fully functional *AFP*-TRE. This AFP-TRE is depicted in Figure 27. Ido et al. (1995) describe a 259 bp promoter fragment (nt -230 to nt +29) that is

specific for HCC. *Cancer Res.* 55:3105-3109. The *AFP* enhancer contains two regions, denoted A and B, located between nt -3954 and nt -3335 relative to the transcription start site. The promoter region contains typical TATA and CAAT boxes. Accordingly, in some embodiments, an *AFP*-TRE comprises nucleotides -3954 to -3335 relative to the *AFP* gene transcription start site and a promoter. In other embodiments, an *AFP*-TRE comprises nucleotides -174 to +29 relative to the *AFP* gene transcription start site. In other embodiments, an *AFP*-TRE comprises nucleotides -3954 to -3335 juxtaposed to -174 to +29 relative to the *AFP* gene transcription start site.

Suitable target cells for adenoviral vectors containing *AFP*-TREs are any cell type that allow an *AFP*-TRE to function. Preferred are cells that express, or produce, *AFP*, including, but not limited to, tumor cells expressing *AFP*. Examples of such cells are hepatocellular carcinoma cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto et al. (1992) *Hepatogastroenterology* 39:282-286), primary gall bladder tumor (Katsuragi et al. (1989) *Rinsko Hoshasen* 34:371-374), uterine endometrial adenocarcinoma cells (Koyama et al. (1996) *Jpn. J. Cancer Res.* 87:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce *AFP*. Especially preferred are hepatocellular carcinoma cells and any of their metastases. *AFP* production can be measured using assays standard in the art, such as RIA, ELISA or Western blots (immunoassays) to determine levels of *AFP* protein production or Northern blots to determine levels of *AFP* mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (i.e., allow an *AFP*-TRE to function).

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a urokinase plasminogen activator TRE. The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently occurring neoplasia and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Transcriptional regulatory elements that regulate uPA and uPAR transcription have been extensively studied. Riccio et al.

(1985) *Nucleic Acids Res.* 13:2759-2771; Cannio et al. (1991) *Nucleic Acids Res.* 19:2303-2308.

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a carcinoembryonic antigen TRE. CEA is a 180,000-Dalton glycoprotein tumor-associated antigen present on endodermally-derived neoplasia of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5' upstream flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the translational start site in the 5' flanking region of the gene, was shown to confer cell-specific activity when the region provided higher promoter activity in CEA-producing cells than in non-producing HeLa cells. Schrewe et al. (1990) *Mol. Cell. Biol.* 10:2738-2748. In addition, cell-specific enhancer regions have been found. WO 95/14100. The entire 5' *CEA* flanking region (containing the promoter, putative silencer, and enhancer elements) appears to be contained within approximately 14.5 kb upstream from the translation start site. Richards et al. (1995); WO 95/14100. Further characterization of the 5' flanking region of the *CEA* gene by Richards et al. (1995) indicated two upstream regions, -13.6 to -10.7 kb or -6.1 to -4.0 kb, when linked to the multimerized promoter resulted in high-level and selective expression of a reporter construct in CEA-producing LoVo and SW1463 cells. Richards et al. (1995) also localized the promoter region to nt -90 and nt +69 relative to the translational start site, with region nt -41 to nt -18 as essential for expression. WO95/14100 describes a series of 5' flanking *CEA* fragments which confer cell-specific activity, such as about nt -299 to about nt +69; about nt -90 to about nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600, nt -6100 to nt -3800. In addition, cell specific transcription activity is conferred on an operably linked gene by the *CEA* fragment from nt -402 to nt +69, depicted in (SEQ ID NO:10). In the above-described CEA-TREs, numbering is relative to the translation start site.

As shown in Example 4, cell type-specific transcription activity is also conferred on an operably linked gene by a CEA promoter fragment from nucleotide (nt) -300 to +70 relative to the CEA gene transcription start site (nucleotides 14161 to 14532 of the sequence depicted in Figure 23 (SEQ ID NO:10)). Accordingly, in some embodiments, an E3-containing adenoviral vector of the invention comprises a CEA-TRE comprising a nucleotide sequence from about 14161 to about 14532 of SEQ ID NO:10), i.e., CN799, shown in Figure 16. This promoter fragment can be used alone or in combination with one or more enhancer fragments derived from a CEA gene 5' flanking region. Thus, as shown in Example 4, cell type-specific transcription activity is also conferred on an operably linked gene by a CEA-TRE comprising a promoter fragment from -300 to +70 relative to the CEA gene transcription start site juxtaposed to a CEA enhancer fragment comprising nucleotides -6072 to -3815 relative to the CEA transcription start site (nucleotides 8392 to 10548 of the sequence depicted in Figure 23 (SEQ ID NO:10)). Accordingly, in some embodiments, an E3-containing adenoviral vector of the invention comprises a CEA-TRE comprising a nucleotide sequence from about 14161 to about 14532 of SEQ ID NO:10) operably linked to a nucleotide sequence from about 8392 to about 10548 of SEQ ID NO:10), i.e., CN798, shown in Figure 16. In other embodiments, a CEA-TRE comprises a CEA promoter fragment from about -300 to about +70 relative to the CEA transcription start site operably linked to a CEA enhancer fragment from about -6072 to about -3815 and about -13.6 kb to about -10.7 kb relative to the CEA gene transcription start site. Any of the CEA-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Thus, any of the CEA-TREs may be used in the invention as long as requisite desired functionality is displayed in the adenovirus vector. The cloning and characterization of CEA sequences have been described in the literature and are thus made available for practice of this invention and need not be described in detail herein.

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a mucin TRE. The protein product of the *MUC1* gene (known as mucin or MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of epithelial cells lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter et al. (1988) *Cancer Rev.* 11-12: 55-

101; and Girling et al. (1989) *Int. J. Cancer* 43: 1072–1076. However, mucin is overexpressed in 75–90% of human breast carcinomas. Kufe et al. (1984) *Hybridoma* 3: 223–232. For reviews, see Hilkens (1988) *Cancer Rev.* 11–12: 25–54; and Taylor-Papadimitriou, et al. (1990) *J. Nucl. Med. Allied Sci.* 34: 144–150. Mucin protein
5 expression correlates with the degree of breast tumor differentiation. Lundy et al. (1985) *Breast Cancer Res. Treat.* 5: 269–276. This overexpression appears to be controlled at the transcriptional level.

Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and ZR-75-1 appears to be regulated at the transcriptional level. Kufe et al. (1984); Kovarik
10 (1993) *J. Biol. Chem.* 268:9917–9926; and Abe et al. (1990) *J. Cell. Physiol.* 143: 226–231. The regulatory sequences of the *MUC1* gene have been cloned, including the approximately 0.9 kb upstream of the transcription start site which contains a TRE that appears to be involved in cell-specific transcription. Abe et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 282–286; Kovarik et al. (1993); and Kovarik et al. (1996) *J. Biol. Chem.*
15 271:18140–18147.

Any *MUC1*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE may contain the entire 0.9 kb 5' flanking sequence of the *MUC1* gene. In other embodiments, the *MUC1*-TREs comprise the following sequences
20 (relative to the transcription start site of the *MUC1* gene): about nt -725 to about nt +31, nt -743 to about nt +33, nt -750 to about nt +33, and nt -598 to about nt +485 (operably-linked to a promoter).

In some embodiments, the E3-containing adenovirus vectors of the invention comprise a c-erbB2/neu TRE. The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a
25 transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the c-erbB2/neu protein is expressed during fetal development, however, in adults, the protein is weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with many human cancers,
30 including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of the c-erbB2/neu protein over-expression have been best studied in breast

and ovarian cancer. c-erbB2/neu protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases. Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer *c-erbB2/neu* expressing cell specific activity. Tal et al. (1987) *Mol. Cell. Biol.* 7:2597–2601; Hudson et al. (1990) *J. Biol. Chem.* 265:4389–4393; Grooteclaes et al. (1994) *Cancer Res.* 54:4193–4199; Ishii et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:4374–4378; Scott et al. (1994) *J. Biol. Chem.* 269:19848–19858.

The cell type-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell type-specific TREs are known in the art, as are methods to identify and test cell specificity of suspected cell type-specific TREs.

Cell status-specific TREs

Cell status-specific TREs for use in the E3-containing adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

An example of a cell status is cell cycle. An exemplary gene whose expression is associated with cell cycle is E2F-1, a ubiquitously expressed, growth-regulated gene, which exhibits peak transcriptional activity in S phase. Johnson et al. (1994) *Genes Dev.* 8:1514–1525. The RB protein, as well as other members of the RB family, form specific complexes with E2F-1, thereby inhibiting its ability to activate transcription. Thus, E2F-1-responsive promoters are down-regulated by RB. Many tumor cells have disrupted RB function, which can lead to de-repression of E2F-1-responsive promoters, and, in turn, de-regulated cell division.

Accordingly, in one embodiment, the invention provides an E3-containing adenoviral vector in which an adenoviral gene (preferably a gene necessary for replication) is under transcriptional control of a cell status-specific TRE, wherein the cell status-specific TRE comprises a cell cycle-activated TRE. In one embodiment, the cell cycle-activated

TRE is an E2F1 TRE. In one embodiment, this TRE comprises the sequence depicted in Figure 21 (SEQ ID NO:8).

Another group of genes that are regulated by cell status are those whose expression is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.* 76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemín and Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to the fact that tumor cells typically grow faster than the endothelial cells that make up the blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1, which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of several genes, including vascular endothelial growth factor, and several genes encoding glycolytic enzymes, including enolase-1. Murine HRE sequences have been identified and characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335. An HRE from a rat enolase-1 promoter is depicted in Figure 20 (SEQ ID NO:7).

Accordingly, in one embodiment, an E3-containing adenovirus vector comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a cell status-specific TRE comprising an HRE. In one embodiment, the cell status-specific TRE comprises the HRE depicted in Figure 20 (SEQ ID NO:7).

Other cell status-specific TREs include heat-inducible (i.e., heat shock) promoters, and promoters responsive to radiation exposure, including ionizing radiation and UV radiation. For example, the promoter region of the early growth response-1 (Egr-1) gene contains an element(s) inducible by ionizing radiation. Hallahan et al. (1995) *Nat. Med.* 1:786-791; and Tsai-Morris et al. (1988) *Nucl. Acids. Res.* 16:8835-8846. Heat-inducible promoters, including heat-inducible elements, have been described. See, for example Welsh (1990) in "Stress Proteins in Biology and Medicine", Morimoto, Tisseries, and Georgopoulos, eds. Cold Spring Harbor Laboratory Press; and Perisic et al. (1989) *Cell* 59:797-806. Accordingly, in some embodiments, the cell status-specific TRE comprises an element(s) responsive to ionizing radiation. In one embodiment, this TRE comprises a 5'

flanking sequence of an Egr-1 gene. In other embodiments, the cell status-specific TRE comprises a heat shock responsive element.

The cell status-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell status-specific TREs are known in the art, as are methods to identify and test cell status specificity of suspected cell status-specific TREs.

Adenovirus genes under control of a target cell-specific TRE

In the E3-containing adenoviral vectors of the present invention, an adenovirus gene, preferably an adenovirus gene essential for replication, is under control of a target cell-specific TRE.

Preferably, the gene(s) is an early gene, such as E1A, E1B, E2, and/or E4. Embodiments in which E3 is under transcriptional control of a target cell-specific TRE have been discussed above. More preferably, the early gene(s) under target cell-specific TRE control is E1A and/or E1B. More than one early gene can be placed under control of an target cell-specific TRE. Example 1 provides a more detailed description of such constructs.

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a *trans*-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* 651:175-208; Flint (1986) *Advances Virus Research* 31:169-228; Grand (1987) *Biochem. J.* 241:25-38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* 31:35-81. The transcription start site of Ad5 E1A is at nucleotide 498 and the ATG start site of the E1A protein is at nucleotide 560 in the virus genome.

The E1B protein functions *in trans* and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40

and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey, Mackay et al. (1993) *Virology* 193:631; Bailey et al. (1994) *Virology* 202:695-706). The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box.

5 The E2 region of adenovirus codes for proteins related to replication of the adenoviral genome, including the 72 kDa DNA-binding protein, the 80 kD precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during
10 late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

 The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable to genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD
15 protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position nt 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a target cell-specific TRE having SpeI ends into the SpeI site in the +-strand would disrupt the
20 endogenous E2 early promoter of Ad5 and should allow target cell-restricted expression of E2 transcripts.

 The E4 gene has a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames
25 (ORFs) 3 and 6 can both perform these functions by binding the 55kD protein from E1B and heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55kD protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than 10^{-6} that of wild type virus. To further restrict viral replication to cells exhibiting a requisite
30 physiological condition or state, E4 ORFs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a

mutant with sequences in which the E1B region is regulated by a target cell-specific TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on a target cell-specific TRE driving E1B.

The major late genes relevant to the subject invention are genes L1, L2, L3, L4, and L5 which encode proteins of the adenovirus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at nucleotides +5986 to +6048.

Transgenes

In addition to conferring selective cytotoxic and/or cytolytic activity by virtue of preferential replication competence in target cells, the adenovirus vectors of this invention can further include a heterologous gene (transgene) under the control of a heterologous TRE. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the target cell-specific cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. aprotinin, papain, proteinase K,

carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN- α , - β , - γ , TNF- α , - β , TGF- α , - β , NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

Preparation of the adenoviral vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Any of the various serotypes of adenovirus can be used in the present invention, such as Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, serotype Ad5 is exemplified herein.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, by recombinant methods, and/or by obtaining the desired sequence(s) from biological sources.

Adenoviral vectors are conveniently prepared by employing two plasmids, one plasmid providing for the left hand region of adenovirus and the other plasmid providing for the right hand region, where the two plasmids share at least about 500 nucleotides of middle region for homologous recombination. In this way, each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent host, providing complementing genes as appropriate. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as lipid-containing agents (e.g., cationic liposomes). Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acids Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHGE3 (Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, including E3.

For manipulation of the early genes, the transcription start site of Ad5 E1A is at nt 498 and the ATG start site of the E1A protein is at nt 560 in the virus genome. This region

can be used for insertion of a target cell-specific TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a nucleotide sequence change resulting in a unique restriction site, one can provide for insertion of target cell-specific TRE at that site. Example 1 provides a more detailed description of an adenoviral vector in which E1A is under target cell-specific TRE control.

A similar strategy may also be used for insertion of a heterologous TRE to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from nt 1636 to nt 1701. By insertion of a heterologous TRE in this region, one can provide for target cell-specific transcription of the E1B gene. By employing the left-hand region modified with a heterologous TRE regulating E1A as the template for introducing a heterologous TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the target cell-specific transcription factors for expression of both E1A and E1B.

Similarly, a heterologous TRE can be inserted upstream of the E2 gene to make its expression target cell specific. The E2 early promoter, mapping in Ad5 from nt 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site. For a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Microbiol. and Immunol.* (1995) 199 (part 3):177-194.

For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at 35609, the TATA box at nt 35638 and the first ATG/CTG of ORF 1 is at nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a heterologous TRE may be introduced upstream from the transcription start site. For the construction of mutants in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:5383-5386) which provide E4 proteins *in trans*

to complement defects in synthesis of these proteins. Alternatively, these constructs can be produced by *in vitro* ligation.

Adenoviral constructs containing an E3 sequence can be generated as described in Example 1, wherein homologous recombination between an E3-containing adenoviral plasmid, for example, BHGE3 (Microbix Biosystems Inc., Toronto) and a non-E3-
5 containing adenoviral plasmid, is carried out.

Alternatively, an adenoviral vector comprising an E3 sequence can be introduced into cells, for example 293 cells, along with an adenoviral construct or an adenoviral plasmid construct, where they can undergo homologous recombination to yield adenovirus
10 containing an E3 region (or portion of E3). In this case, the E3-containing adenoviral vector and the adenoviral construct or plasmid construct contain complementary regions of adenovirus, for example, one contains the left-hand and the other contains the right-hand region, with sufficient sequence overlap as to allow homologous recombination.

Alternatively, an E3-containing adenoviral vector of the invention can be
15 constructed using other conventional methods including standard recombinant methods (e.g., using restriction nucleases and/or PCR), chemical synthesis, or a combination of any of these. Further, deletions of portions of the E3 region can be created using standard techniques of molecular biology.

Host cells comprising the adenoviral vectors of the invention

20 The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Prokaryotic host cells include bacterial cells, for example, *E. coli* and mycobacteria.
25 Among eukaryotic host cells are yeast, insect, avian, plant and mammalian cells. Host systems are known in the art and need not be described in detail herein.

Compositions of the invention

The present invention also provides compositions, including pharmaceutical compositions, containing an adenoviral vector(s) described herein. Such compositions
30 (especially pharmaceutical compositions) are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an

individual. Pharmaceutical compositions, comprised an adenoviral vector of this invention in a pharmaceutically acceptable excipient (generally an effective amount of the adenoviral vector), are suitable for systemic or localized administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing (1990).

Pharmaceutical compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

Other compositions are used, and are useful for, detection methods described herein. For these compositions, the adenoviral vector usually is suspended in an appropriate solvent or solution, such as a buffer system. Such solvent systems are well known in the art.

Kits of the invention

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow detection of the presence of cells which allow a target cell-specific TRE to function, including, for example, neoplastic cells, in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Methods using the adenovirus vectors of the invention

The adenoviral vectors of the invention can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

In one embodiment, methods are provided for conferring selective cytotoxicity in target cells (i.e., cells which allow a target cell-specific TRE to function) comprising contacting the cells with an adenovirus vector described herein, such that the adenovirus vector enters the cell (and subsequently replicates). Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ³H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus specific for mammalian cells which allow a target cell-specific TRE to function. These methods entail combining an adenovirus vector with mammalian cells, whereby said adenovirus enters the cell and is propagated.

The invention further provides methods of suppressing tumor cell growth comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell (and/or whereby introduction of the adenoviral vector results in suppression of tumor growth). Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ³H-thymidine incorporation assay, or counting tumor cells.

The invention also includes methods for detecting target cells in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. For these methods, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. A suitable biological sample is one in which target cells, for example, cancerous cells, may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells exhibiting a requisite physiological state, such as cells within a solid tumor which are under hypoxic conditions, or cancerous or non-cancerous cells of a particular cell type, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions, such as selective enrichment, and/or solubilization. In these methods, target cells can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles

per cell). Propagation can also be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective.

5 The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs; polynucleotide constructs complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMA™, DOTAP™, and polyamines.

10 If an adenoviral vector is packaged into an adenovirus, the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virology* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus (or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

20 The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art (such as calcium phosphate precipitation or electroporation), direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

25 If used as a packaged adenovirus, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14} . The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to about 1000 μg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, whether systemically or locally, depending upon the

30

intended use and the immune response potential of the host, and may also be administered as multiple, simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

EXAMPLE 1

Construction of target cell-specific adenoviral constructs containing an E3 region

Target cell-specific adenoviral vectors were constructed by first generating target cell-specific adenoviral vectors and/or adenoviral plasmid vectors lacking an E3 region, then recombining these vectors with E3-containing adenoviral constructs.

With respect to adenoviral constructs (as opposed to precursor plasmid constructs), it is understood that "CN" and "CV" designations may be used interchangeably. For example, CN787 and CV787 refer to the same adenoviral construct.

Generation of adenoviruses and adenoviral plasmid vectors that contain target cell-specific TREs driving expression of E1A and/or E1B

A human embryonic kidney cell line, 293, efficiently expresses E1A and E1B genes of Ad5 and exhibits a high transfection efficiency with adenovirus DNA. For these experiments, 293 cells were co-transfected with one left end Ad5 plasmid and one right end Ad5 plasmid. Homologous recombination generates adenoviruses with the required genetic elements for replication in 293 cells which provide E1A and E1B proteins *in trans* to complement defects in synthesis of these proteins.

The plasmids to be combined were co-transfected into 293 cells using cationic liposomes such as Lipofectin (DOTMA:DOPE™, Life Technologies) by combining the two plasmids, then mixing the plasmid DNA solution (10 µg of each plasmid in 500 µl of minimum essential medium (MEM) without serum or other additives) with a four-fold

molar excess of liposomes in 200 μ l of the same buffer. The DNA-lipid complexes were then placed on the cells and incubated at 37°C, 5% CO₂ for 16 hours. After incubation the medium was changed to MEM with 10% fetal bovine serum and the cells are further incubated at 37°C, 5% CO₂, for 10 days with two changes of medium. At the end of this time the cells and medium were transferred to tubes, freeze-thawed three times, and the lysate was used to infect 293 cells at the proper dilution to detect individual viruses as plaques.

Plaques obtained were plaque purified twice, and viruses were characterized for presence of desired sequences by PCR and occasionally by DNA sequencing. For further experimentation, the viruses were purified on a large scale by cesium chloride gradient centrifugation.

CN739

An adenovirus vector in which expression of the E1A gene is under control of the *PB*-TRE and expression of the E1B gene is under control of the prostate specific antigen transcriptional regulatory element (*PSA*-TRE) was constructed as follows. The *PSA*-TRE region has been described in detail in, inter alia, U.S. Patent Nos. 5,648,478 and 5,698,443; Lundwall (1989) *Biochim. Biophys. Res. Commun.* 161:1151-1159; and Zhang et al. (1997) *Nucleic Acids Res.* 25:3143-50.

The PinAI *PB*-TRE fragment was inserted into CN125 digested with PinAI, which cleaves just upstream of E1A, to create construct CN257, which is a plasmid containing a *PB*-TRE (from -426 to +28 relative to the rat probasin gene transcription start site) operably linked to the E1A gene and a *PSA*-TRE (from -5322 to -3738 and -541 to +12 relative to the human *PSA* gene transcription start site) operably linked to the E1B gene.

Adenoviruses that contain two heterologous TREs were generated by homologous recombination in 293 cells. Briefly, 5 μ g of CN257 and 5 μ g of BHG10, which contains the right hand end of Ad5, was co-transfected into 293 cells. The cells were overlaid with medium, and infectious virus, generated by *in vivo* recombination, was detected by cytopathic effect and isolated. Plaque-purified stocks of an adenovirus vector, designated CN739, were established. The structure of the recombinant virus was characterized by PCR, restriction endonuclease digestion and Southern blot. The viral genome of CN739

has the E1A transcription unit of Ad5 under the control of *PB*-TRE while E1B is under the control of *PSA*-TRE.

CN732

5 CN732 (also referred to as CV732) which contains AFP-TRE driving expression of the E1A gene, was constructed by PCR as described below. CN732 contains the AFP enhancer domain (-3954 to -3335) and the promoter (-174 to +29) cloned into an engineered AgeI site at nt 547 of Ad5 to drive Ad5 E1a expression.

pXC.1 contains the left 16 mu of Ad5. pBHGE3 from Microbix, Inc., Toronto, Canada contains the circularized Ad5 genome with an E1 deletion extending to the packaging signal. Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806; and McKinnon et al. (1982) *Gene* 19:33-42. A platform plasmid (CP124) with two unique restriction sites was constructed from pXC.1. CP124 has an AgeI site at bp 547 between the E1a mRNA cap site and E1a translation initiation site, and an EagI site at bp 1681 between the E1b promoter and E1b mRNA cap site. To construct viruses that preferentially replicate in AFP⁺ cells, a composite AFP TRE of the enhancer and promoter was assembled by overlap PCR and cloned into CP124. The enhancer element (-3856 to -3267) was amplified from human genomic DNA (Clontech, Palo Alto, CA) with primers 5' GTGACCGGTG CATTGCTGTG AACTCTGTA 3' (39.055B) (SEQ ID NO:); and 5' ATAAGTGGCC TGGATAAAGC TGAGTGG 3' (38.055D) (SEQ ID NO:).

20 The promoter (-163 to +34) was amplified with primers 5' GTCACCGGTC TTTGTTATTG GCAGTGGT 3' (39.055J) (SEQ ID NO:); and 5' ATCCAGGCCA CTTATGAGCT CTGTGTCCTT 3' (39.055M) (SEQ ID NO:). Underlined regions in primers 39.055B and 39.055J indicate AgeI sites. The two products were annealed and used as the template for PCR with primers 39.055B and 39.055J. This overlap product was digested with PinAI (an AgeI isoschizomer) and ligated to PinAI cut CP124.

25

The resulting plasmid, CN219, contains the AFP TRE driving the E1A gene. CN732 was generated via homologous recombination by cotransfecting 293 cells with CN219 with pBHG10. pBHG10 is a plasmid that contains all of the Ad5 sequences except the E1 region as well as a deletion in the E3 region from Ad5 bp 28133 to 30818.

30

CN733

CN733 (also referred to as CV733) which contains two AFP-TREs driving expression of the E1A and E1B genes, was constructed by using the following two PCR primers to amplify the enhancer/promoter element described above (-3954 to -3335 and -174 to +29):

5' TATCGGCCGG CATTGCTGTG AACTCT 3' (39.077A) (SEQ ID NO:1); and

5' TTACGGCCGC TTTGTTATTG GCAGTG 3' (39.077C) (SEQ ID NO:2).

The PCR product was digested with EagI and ligated into similarly cut CN219. The resulting plasmid, CN224, contains two identical AFP regulatory elements, one each modulating expression of the E1A gene and the E1B gene. CN733 was generated by homologous recombination in 293 cells by cotransfecting CN224 and BHG10.

CN796

Adenoviral vector CN796 comprises a composite TRE comprising an HRE and a PSA-TRE, the PSA-TRE comprising a PSA enhancer from -5322 to -3738 fused to a PSA promoter from -541 to +12. This PSA-TRE is derived from adenoviral vector CN706. Rodriguez et al. (1997) *Cancer Research* 57:2559-2563.

CN796 was made by co-transfecting CN515 with pBHG10. CN515 was constructed by inserting a 67 base pair fragment from HRE eno1 (Jiang et al. (1997) *Cancer Research* 57:5328-5335) (SEQ ID NO:7; Figure 20) into CN65 at the BglII site. CN65 is a plasmid containing an enhancer and promoter from the human PSA gene, consisting of an enhancer from -5322 to -3738 fused to a PSA promoter from -541 to +12. This is the PSA-TRE contained within plasmid CN706.

CN532

CEA-TRE-containing adenoviral vector CN532 was generated as follows. The native E1A promoter was deleted (as a 64 bp fragment) from CN124 (wild-type left-hand end of Ad5) to generate CN306. CN143 is a pBluescript (Stratagene, La Jolla, California) derivative containing a PSA-TRE fragment. The PSA-TRE fragment from CN143 was then inserted upstream of the promoter-less E1A coding segment of CN306 to generate CN321.

CN532 was constructed from CN321 by replacing the *PSA*-TRE of CN321 with a CEA promoter. The CEA promoter was amplified from human genomic DNA by PCR using two primers:

5' GATCTCGAGA CCCGGGACCC TGCTGGGTTT C 3' (76.13.1) (SEQ ID NO:3); and

5' GATCACCGGT GCTTGAGTTC CAGGAACGTT TTG 3' (76.13.2) (SEQ ID NO:4).

The PCR product was enzymatically digested with *Pin*AI, then ligated to *Pin*AI-cut CN321 vector portion. The CEA promoter fragment amplified by these primers corresponds to -300 to +70 relative to the CEA gene transcription start site.

CN525

As with CN532, CN525 was generated from CN321 by replacing the *PSA*-TRE with a CEA promoter and enhancer. The CEA enhancer was amplified from human genomic DNA by PCR using two synthetic oligonucleotide primers:

5' GATCAACCGG TACCGACTTC TGTAGCTTTG GGAAGG 3' (76.13.3) (SEQ ID NO:5); and

5' GATCCTCGAG CCCGGGTTC AAGCAATTCTC CTGC 3' (76.13.4) (SEQ ID NO:6).

The PCR product was cut with *Pin*AI, ligated to the CEA promoter fragment described above, and ligated to *Pin*AI-cut CN321. The orientation of the enhancer fragment is 3' to 5' relative to the promoter. The CEA enhancer fragment amplified by 76.13.3 and 76.13.4 corresponds to -6072 to -3815 relative to the CEA gene transcription start site. Thus, CN525 contains a CEA-TRE comprising a CEA enhancer from -6072 to -3815 juxtaposed to a CEA promoter from -300 to +70 relative to the CEA gene transcription start site.

Generation of recombinant adenoviruses containing E3

Adenovirus containing E3 were generated by homologous recombination in 293 cells. Briefly, CN257 was co-transfected with pBHGE3 (Microbix) into 293 cells. The cells were overlaid with media, and infectious virus generated by *in vivo* recombination was detected by cytopathic effect and isolated. Plaque-purified stocks of an adenovirus vector, designated CN787, were established. The structure of the recombinant virus was characterized by PCR, restriction endonuclease digestion and Southern blot. The resulting

recombinant virus, CN787 (also referred to as CV787), is a full-length Ad5 with a *PB*-TRE driving the expression of E1A and a *PSA*-TRE driving expression of E1B, and which also contains an E3 gene. Thus, CN787 is analogous to CN739, except that it contains an E3 region. This construct is depicted schematically in Figure 2.

5 Using this method, CN790, CN797, CN798, CN799, and CN802 (also referred to as CV790, CV797, CV798, CV799 and CV802, respectively) were generated by recombination between BHGE3 and CN733, CN796, CN532, CN525, and pXC.1, respectively. Plasmid pXC.1 (Microbix) is wild-type adenovirus Type 5. CN790 is the same as CN733 but with the addition of the Ad5 full-length E3 region. CN790 is depicted
10 schematically in Figure 11; CN798, CN799, and CN802 are depicted schematically in Figure 16; and CN797 is depicted schematically in Figure 19.

Structure, Genomic Stability, and Self-Inactivation of Viruses

15 Virus genetic structures were confirmed by PCR and Southern blot analysis.

Southern Blot Analysis. 10 ng of viral DNA (QIAmp blood kit, Qiagen) was digested with AflII or AflII and ClaI. DNA was fractionated through a 1% agarose gel and transferred by capillary transfer to a Nytran nylon membrane (Schleicher and Schuell). Viral DNA was probed with [α -³²P] dCTP labeled PCR products specific for E1a or E1b
20 sequences. The E1A probe was made by PCR from CN706 DNA amplifying a 938bp fragment of Ad5 DNA with primers

5' GAGACATATT ATCTGCCACG G 3' (18.184.1) (SEQ ID NO:); and

5' CGTTAAGCAA GTCCTCGATA C 3' (18.184.2) (SEQ ID NO:),

and the E1b probe was made from CN706 DNA using primers

25 (5' TTGGTTTTGG AGGTTTCTGT GGGG 3' (46.135B) (SEQ ID NO:); and

(5' AAAGGCCACC CTATCCTCCG TATC 3' (46.135E) (SEQ ID NO:) (881bp product).

Blots were hybridized overnight at 45°C in ZipHyb solution (Ambion), washed two times in 2XSSC, 0.1%SDS at room temperature and two times in 0.1XSSC, 0.1 %SDS at 65°C.

Blots were visualized by exposure in a GS-525 Molecular Imager (BioRad Laboratories).

30 The proportion of CN733 or CN790 deletion mutant (Δ E1a CN733 or Δ E1a CN790) in each stock was estimated by determining the amount of radioactivity in bands

corresponding to the CN733 or CN790 (5.2 kb band, AflII digest and E1b probe) and the Δ E1a CN733 or Δ E1a CN790 (3.2 kb band) using the Molecular Imager and Multi-Analyst imaging software (Bio-Rad, version 1.0.2).

Results. Viral DNA was digested with AflII to release the E1 region and probed with [α -³²P] dCTP labeled DNA specific for the E1a gene (Figure 29A) and the E1b gene (Figure 29B). The expected fragment sizes are shown in Table 1.

Table 1

<u>Virus</u>	Expected Fragment Sizes (kb)	
	<u>E1A Probe</u>	<u>E1B Probe</u>
	<u>AflII</u>	<u>AflII</u>
CN702	3.5	3.5
CN732	4.4	4.4
CN733	5.2	5.2
Δ E1a CN733	-	3.2
CN734	4.4	4.4
CN739	5.7	5.7
CN790	5.2	5.2
Δ E1a CN790	-	3.2

All the viruses had the expected structure and CN702, CN732, CN734 and CN739 were stable through three passages. However, preparations of CN733 and CN790 showed the expected virus, indicated by a 5.2 kb band, but also contained an additional 3.2kb band (Figure 29B). This band did not appear in Southern blots probed with E1a but did appear in Southern blots probed with E1b. The DNA of individual clones of CN733 and CN790 were cloned and their DNAs sequenced. DNA sequence analysis of individual clones indicated homologous recombination occurred between the two AFPs and the AFP DNA segments during propagation, resulting in the deletion of the E1a gene and one copy of the AFP TRE. These deletion viruses were named Δ E1a CN733 and Δ E1a CN790 respectively. The rate of accumulation of Δ E1a CN733 and Δ E1a CN790 in stocks of CN733 and CN790 was evaluated by performing Southern blot analysis on several viral stocks prepared in series. Data presented in Figure 30 indicate that Δ E1a CN790 was 7% at

the first passage and accumulated to about 14% of the total viral particles by the third passage in 293 cells. $\Delta E1a$ CN790 accumulated rapidly in cells that did not select against the replication defective mutant like 293 cells or in cells that had been infected at an MOI = 10. However, CN790 prepared at low MOI = 0.1 in AFP⁺ HepG2 cells that did not support replication defective adenovirus replication was devoid of the deletion mutant after three passages (Figure 30).

EXAMPLE 2

In vitro and in vivo characterization of CN787, an E3-containing adenoviral construct comprising a PB-TRE driving expression of E1A and a PSA-TRE driving expression of E1B

Cells and Culture Methods

LNCaP cells were obtained at passage 9 from the American Type Culture Collection (Rockville, MD). LNCaP cells were maintained in RPMI 1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS; Interger Corp.), 100 units/mL of penicillin, and 100 units/mL streptomycin. LNCaP cells being assayed for luciferase expression were maintained in 10% strip-serum (charcoal/dextran treated fetal bovine serum to remove T3, T4, and steroids; Gemini Bioproduct, Inc., Calabasas, CA) RPMI. The cells were periodically tested for the production of PSA which was consistently above 20 ng/mL per day.

Transfections of LNCaP Cells

For transfections, LNCaP cells were plated out at a cell density of 5×10^5 cells per 6-cm culture dish (Falcon, NJ) in complete RPMI. DNAs were introduced into LNCaP cells after being complexed with a 1:1 molar lipid mixture of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP[™]; Avanti Polar Lipids, AL) and dioleoyl-phosphatidylethanolamine (DOPE[™]; Avanti Polar Lipids, AL); DNA/lipid complexes were prepared in serum-free RPMI at a 2:1 molar ratio. Typically, 8 μ g (24.2 nmole) of DNA was diluted into 200 μ L of incomplete RPMI and added dropwise to 50 nmole of transfecting, lipids in 200 μ L of RPMI with gentle vortexing to insure homogenous mixing of components. The DNA/lipid complexes were allowed to anneal at room temperature for 15 minutes prior to their addition to LNCaP cells. Medium was removed from LNCaP

cells and replaced with 1 mL of serum-free RPMI followed by the dropwise addition of DNA/lipid complexes. Cells were incubated with complexes for 4-5 hours at 37°C, 5% CO₂. Medium was removed and cells washed once with PBS. The cells were then trypsinized and resuspended in 10% strip-serum RPMI (phenol red free). Cells were replated into an opaque 96-well tissue culture plate (Falcon, NJ) at a cell density of 40,000 cells/well per 100 µL media and assayed. Varying amounts of drugs (e.g. androgens and anti-androgens) were added 16 hours later and assayed for luciferase activity 32 hours thereafter.

Plaque assays

To determine whether the adenoviral constructs described above replicate preferentially in prostate cells, plaque assays were performed. Plaquing efficiency was evaluated in the following cell types: prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). LNCaP cells express both androgen receptor and PSA, while the other cell lines tested do not. 293 cells serve as a positive control for plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. For analyzing constructs comprising a CEA-TRE, cells that allow a CEA-TRE to function, such as NCIH508, LoVo, SW1463, MKN1, MKN28, MKN45 and cells that do not allow such function, such as HuH7, HeLa, PA-1, or G361, are used. For analyzing constructs comprising an AFP-TRE, cell lines which express AFP, such as HepG2 and Hep3B, are compared with cell lines which do not express AFP, including HBL-100, OVCAR-3, and LNCaP. The plaque assay was performed as follows: Confluent cell monolayers were seeded in 6-well dishes eighteen hours before infection. The monolayers were infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the media was removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques were scored two weeks after infection. CN702 has no modifications in its E1 region, but is devoid of E3, and thus serves as a wild type control for left end modifications lacking E3. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997) *Cancer Res.* 57:2559-2563.

In vitro characterization of CN787

Various parameters of CN787 were measured, including cell type specificity, plaque size, extracellular virus yield, kinetics of cell killing, and total viral yield per cell.

Cell type specificity of CN787

5 To determine whether adenoviral construct CN787, described above, replicate preferentially in prostate cells, plaque assays were performed as described above. The results are shown in Figure 3. The results are also summarized in Figure 2, where they are expressed as percent of wild-type adenovirus plaque-forming units (PFU) per ml. The average titer of duplicate samples for the viruses tested. The titer for a particular virus in
10 all cell lines was normalized to its titer on 293 cells. Once the titers on a cell type were normalized to 293 cells, the normalized numbers of the recombinant viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702. The ratio of specificity of CN787 to that of CN702 is
15 10,000:1.

These results indicate that CN787 exhibits a specificity for prostate cells comparable to that of CN739.

Viral production

20 While CN787 and CN739 demonstrate comparable cell-type specificity, CN787 gave much larger plaques than did CN739, as shown in Figure 4.

One measure of viral production is extracellular virus yield per cell. CN787 and CN739 were plated on LNCaP cells at 2 PFU/cell, and plated at 10^6 cells per well. At various times after infection, the cell supernatant was tested on 293 cells to measure the
25 number of plaque-forming units. The results, presented in Figure 5, show that CN787 gave a significantly higher viral yield than did CN739.

When total virus yield was examined, it was found that, at 96 hours post infection, CN787 yielded at least one log higher number of plaque forming units per cell, as shown in Figure 6. The total viral yield per cell is the extracellular viral yield per cell plus the
30 intracellular viral yield per cell. CN787 yielded more extracellular virus per cell than did CN739, indicating that that E3 mediates a fast release of viruses from the infected cells. The total viral yield per cell from cells infected with CN787 is higher than that of cells

infected with CN739, suggesting that CN787-infected cells produce more viruses. The higher total yield could be due to virus replication/DNA replication, or a higher efficiency of viral DNA packaging.

To examine the kinetics of cell killing by CN787, two different assays for cell viability, the MTT assay and the trypan blue dye exclusion assay, were performed. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed as follows. 293 cells were infected with CN787 or CN739 at a concentration of 2 PFU/cell and plated at a cell density of 10^4 cells per well at various times post-infection. At the indicated times post-infection, 25 μ l MTT (Sigma Chemical Co., St. Louis, MO) in PBS at a concentration of 5 mg/ml was added to each well. After 2 hours, lysis buffer (20% sodium dodecyl sulfate in 50:50 dimethyl formamide:double-distilled H_2O , pH 4.7) was added to each well and plates were incubated overnight at 37°C. Plates were read on a microplate reader at 570 nm. Samples were in triplicate. The results, shown in Figure 7, indicate that when cells were infected with CN787, the absorbance dropped sharply after 4 days post infection, and by 7 days post-infection, virtually all cells were killed. In contrast, cells were killed less rapidly by CN739, which is analogous to CN787 but lacks an E3 region.

As a second assay for cell viability, trypan blue exclusion was performed. At various times after infection, a sample of cells 10^6 LNCaP cells was removed. Cells were separated from the medium and trypsinized. Trypan blue (Gibco BRL, Gaithersburg, MD) was added to a final concentration of 0.02%. Cells were counted on a hemacytometer. As with the MTT assay, the trypan blue exclusion assay (Figure 8) showed that a reduction in viability to 50 % viability of LNCaP cells was achieved in 3 days by CN787, compared with 6.5 days by CN739. Thus, by two different assays, LNCaP cells were killed more rapidly by CN787 than by CN739.

In vivo characterization of CN787

An especially useful objective in the development of prostate-specific adenoviral vectors is to treat patients with prostate carcinoma. An initial indicator of the feasibility is to test the vectors using a technique known in the art, such as testing the vectors for cytotoxicity against cancer cells such as tumor xenografts grown subcutaneously in Balb/c nu/nu mice. Mice are given subcutaneous injections with 1×10^7 carcinoma cells, such as

LNCaP or HepG2, in PBS. Tumor cells can be tested for secretion of a tumor-specific product, such as, for example, PSA or AFP, by assaying for the product in serum using standard assays (for example, ELISA). Alternatively, as described below, tumor volume can be measured.

LNCaP nude mouse xenografts were challenged with a single intratumoral dose (1×10^4 particles/mm³ tumor) of either CN787, a vector containing the E3 gene, or CN739, a vector lacking the gene. A third group of tumors was treated with buffer alone. The tumors were monitored weekly for six weeks and their relative volume was graphed against time. The results are shown in Figure 9. Error bars represent the standard error for each sample group. The initial average tumor volume for CN787 treated animals (n=7) was 270 mm³, 295 mm³ for CN739 treated (n=7), and 270 mm³ for buffer treated (n=7). The data suggest that CN787 kills tumor cells more effectively than CN739. On average, tumors challenged with CN739 remained the same size throughout the course of the experiments. Those treated with vehicle alone more than doubled in size. Tumors challenged with CN787 were reduced in size by 40% by week 5. The Students T-test indicates that the difference in tumor size between CN787 and CN739 treated tumors was statistically significant from week 3 through the end of the experiment (p = 0.00209).

Figure 10 presents the results of regimen optimization efforts. LNCaP nude mouse xenografts were challenged with 5×10^{10} CN787 viral particles per day for 5 days (total of 2.5×10^{10} particles CN787) (p = 1.47×10^{-5}) or 1×10^{11} CN787 viral particles per day for 3 days (total of 3×10^{11} CN787 viral particles)(p = 9.2×10^{-7}).

EXAMPLE 3

In vitro and in vivo characterization of CN790, an E3-containing adenoviral construct comprising AFP-TREs driving expression of E1A and E1B

In vitro characterization of CN790

To determine whether adenoviral construct CN790, described above, replicates preferentially in liver cells, plaque assays were performed as described in Example 2. The results, shown in Figure 12, are expressed as percent of wild-type (CN702) adenovirus plaque-forming units (PFU) per ml. The average titer of duplicate samples for the viruses tested. The titer for a particular virus in all cell lines was normalized to its titer on 293

cells. Once the titers on a cell type were normalized to 293 cells, the normalized numbers of the recombinant viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702.

5 CN790 showed a plaquing efficiency comparable to that of CN733 on HepG2 and Hep3B cells. The results indicate that CN790 demonstrates specificity for hepatic cells comparable to that of CN733.

Virus yield assay. Twenty-four well dishes (Falcon) were seeded with 200,000
10 cells per well of Hep3B, HepG2, LNCaP, OVCAR-3, or SK-Hep-1 cells 24 hr prior to infection. Cells were infected at an MOI of 2 PFU/cell for three hours in serum-free media. After the infection was complete, the virus containing media was removed, monolayers were washed three times with PBS, and one ml of complete media was added to each well. 72 hrs post infection, cells were scraped into the culture medium and lysed by three cycles
15 of freeze-thaw. Two independent infections of each virus cell-combination were titered in duplicate on 293 cells.

Results. The addition of the Ad E3 region did not alter virus cell tropism. Cell lines were infected with either CN790 or CN802 (wt Ad5) at a multiplicity of infection (MOI) of 2 PFU/cell. Progeny virus yield was determined 72 hrs after infection by plaque
20 assay on 293 cells (Figure 32). In AFP⁺ cells (Hep3B, HepG2) CN790 replicated similarly to the wild-type control; only a 2-6 fold difference in virus yield was observed. However, CN790 replicated poorly in all AFP⁻ cells tested (LNCaP, OVCAR-3, SK-Hep-1), producing a 10⁵- to 10⁶-fold lower virus yield than CN802. These data indicate that CN790 replicates efficiently in AFP⁺ cells, but is significantly attenuated in AFP⁻ cells.
25 Comparison of CN733 and CN790 in the one-step growth curve in Hep3B and OVCAR-3 cells showed CN790 had the same level of specificity as CN733.

Cytopathic effect of CN790

Cytopathic effect (CPE) assay was carried out to visually assess the specificity of
30 CN790 cytolytic activity. Six well dishes (Falcon) were seeded at 300,000 cells per well with HepG2, Hep3B, Huh-7, LNCaP, OVCAR-3 and SK-Hep-1 cells 24 hr prior to

infection. Cells were infected with CN790 or CN802 at an MOI of 10 PFU/cell, except for SK-Hep-1 cells, which were infected at an MOI of 20 PFU/cell. Virus adsorption was for three hours in 1 ml of serum-free media. Following adsorption, the virus containing media was removed and replaced with 3 ml of RPMI, 10% FBS. Seven days post infection, cells were stained with crystal violet.

Three AFP⁺ cell lines (Hep3B, HepG2, Huh-7) and three AFP⁻ cell lines (LNCaP, OVCAR-3, SK-Hep-1) were mock infected (control) or infected with either CN790 or CN802 (wt Ad5) at an MOI of 10 PFU/cell. SK-Hep-1 cells were infected at an MOI of 20 PFU/cell. Cytopathic effect (CPE) seven days post-infection was monitored by staining remaining cells with crystal violet. The results are shown in Figure 33.

The results indicate that CN790 caused complete CPE in the three AFP expressing cells, but caused little CPE in the three AFP⁻ cells tested, including the AFP⁻ hepatoma line SK-Hep-1. In contrast, wt Ad5 caused significant CPE in all cell lines tested regardless of AFP status. Thus, the cytolytic activity of CN790 is specific for AFP⁺ cell lines and CN790 causes little toxicity to AFP⁻cells.

In vivo characterization of CN790

The effect of CN790 on HepG2 tumors was tested using the tumor xenograft assay system. Six to eight week old athymic Balb/c *nu/nu* mice were obtained from Simonson Laboratories (Gilroy, CA). Xenografts were established by injecting 1-3 x 10⁶ Hep3B or HepG2 cells subcutaneously near the small of the back suspended in 100 µl of RPMI. When tumors reached between 200 µl and 300 µl, mice were randomized and treated with 100 µl of CN790 by tail vein injection. Two treatment regimens were tested. First, Hep3B tumors were treated with 4 x 10⁹ PFU of CN790 or vehicle alone (PBS + 10% glycerol) on days 1, 4 and 8. Second, HepG2 tumors were treated with 5 x 10⁸ PFU of CN790 or vehicle on days 1, 2, 3, 4, 5, 8, 9, 10, 11, and 12. Tumors were measured in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width (mm)²]/2. Animals were sacrificed when tumor burdens became excessive. Serum was harvested weekly by retro-orbital bleed.

HepG2 tumors. On days 1-5 and 8-12 after introducing the HepG2 cells into the mice, CN733, CN739, or CN790, at a concentration of 1x10¹⁰ particles in a volume of 50

µl, or, as a control, vehicle alone, was injected into the mice intravenously. There were 10 mice per test group and in the vehicle-only control group. The results are shown in Figure 13. On average, tumors treated with CN733 or CN790 four weeks after treatment were 857 mm³ and 256 mm³, respectively. Tumors treated with either CN739 or vehicle were 1521 mm³ and 1601 mm³, respectively. Even though both CN733 and CN790 were able to delay tumor progression, CN790 produced a greater tumor response. Both CN733- and CN790-treated tumors increased in size from four to six weeks after the start of treatment. As expected, CN739, a prostate-specific virus, had no effect on tumor growth. CN739-treated tumors, unlike CN733- and CN790-treated tumors, appeared well vascularized. In contrast, CN733- and CN790-treated tumors had a mottled appearance indicative of tumor necrosis.

Hep3B tumors. Subcutaneous Hep3B xenografts in nude mice were treated with intravenously administered CN790. Mice were treated with either 4 x 10⁹ PFU of CN790 (particle/PFU = 20/1) or vehicle alone on days 1, 4, and 8 via tail vein injection. There were 7 mice per test group and 8 in the vehicle-only control group. Tumor size and the concentration of serum AFP were monitored weekly. Control buffer treated mice were sacrificed at four weeks due to excessive tumor burden. Average tumor volumes and serum AFP levels were normalized to 100% at day 1. The results are shown in Figure 34A. The data demonstrate significant tumor killing from CN790. At day 29, the average relative tumor volume of CN790 treated mice was 142%, whereas vehicle treated was 899%. At day 43, one mouse was tumor-free and two others exhibited tumor regression out of seven total animals. Statistical analysis of the results was conducted using the unpaired, two-tailed *t*-test. The differences in mean relative tumor volumes between CN790 treated and buffer treated tumors are significant at days 15, 22 and 29 (*p* < 0.05).

Serum AFP levels in mice treated with CN790

Besides tumor volume, another measure of the efficacy of treatment of tumors is serum concentration of a tumor marker. Serum AFP levels were measured at various times after treatment with adenoviral vectors. The results are shown in Figure 14. HepG2 mice (i.e., mice harboring a HepG2 xenograft) treated as described above with either CN733 or CN790 had average serum AFP concentrations of 214 µg/ml and 79 µg/ml, respectively, at four weeks after the start of treatment. In contrast, mice treated with either vehicle or CN739 had serum AFP concentrations of 443 µg/ml and 619 µg/ml, respectively. In

general, CN733 and CN790 slowed the rise in serum AFP concentration compared to CN739 or buffer. The difference in mean AFP concentration between vehicle-treated and CN733-treated and between vehicle-treated and CN790-treated is significant beginning at week 4.

5 The levels of serum AFP in treated Hep3B mice provide additional evidence of CN790 antitumor activity. At day 29, CN790 treated mice had an average serum AFP concentration of 269%, and vehicle treated mice were 2161%. At day 43, the concentration in CN790 treated mice was 110%. Three mice had decreased serum AFP levels after dosing (Figure 34B). Statistical analysis of the results was conducted using the unpaired,
10 two-tailed *t*-test. The differences in mean serum AFP concentrations between CN790 treated and buffer treated tumors are significant at days 15, 22 and 29 ($p < 0.05$).

In summary, intravenously administered CN790 is more effective at delaying the growth of HepG2 tumors and slowing the rise of serum AFP levels in a nude mouse model for primary liver cancer, when compared to E3⁻ CN733. Furthermore, CN790 not only
15 delayed the growth of Hep3B tumors but eradicated the tumor in one of the mice tested. The greater anti-tumor effective, E3⁺, CN790 indicates that adenoviral vectors that contain an E3 region or portion of E3 are more cytotoxic than those that are deleted for E3. Cells infected with E3-containing virus also release more progeny virus and can be lysed sooner than cells infected with E3-deleted viruses.

20

Mechanism of Tumor Cytolysis by CN790

Immunohistochemistry

Hep3B tumors were established in *nu/nu* mice. Three mice treated with buffer and
25 three mice treated with CN790 (4×10^9 PFU/dose) on days 1, 4, and 8 were sacrificed on day 9. Tumors were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned using standard procedures. To detect adenovirus, tissue sections were blocked with normal rabbit serum (Biogenex, San Carlos, CA) for 20 min and incubated with goat anti-Ad antibody (Biodesign International, Kennebunkport, ME) diluted 1:200 in PBS for
30 30 min. Alkaline phosphatase staining was performed using Super Sensitive streptavidin-biotin alkaline phosphatase reagents and Fast Red chromogen (Biogenex) as suggested by

the manufacturer. Sections were counterstained with Gill's hematoxylin and mounted with Gel Mount (Biomedica, Foster City, CA) aqueous mounting media.

Apoptosis Assay

Apoptotic cells were detected using TdT labeling (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, Intergen, Purchase, NY). Sections were pretreated with 20 µg/ml of proteinase K for 15 min, counterstained with methyl green and mounted with Permount (Fisher). To evaluate the number of apoptotic cells in virus-treated and un-treated control tumors, the tumors were sectioned through their largest diameter and slices were prepared from each half of the resected tumor. The apoptotic index was estimated by the number of positive cells/100X field, eight sections per tumor. A minimum of five 100X fields were counted for each section. The mean of the sections was used for the apoptotic assay, and representative sections presented.

Results. To investigate mechanisms of CN790 tumor destruction, Ad5 hexon staining for virus replication and the apoptotic index by TdT labeling of treated tumors was determined. Virus replication in Hep3B tumors was confirmed by immunohistochemical staining of tumor sections using polyclonal antibodies to Ad5 hexon. Ad5 hexon is not found immediately or several days after intravenous virus administration. However, Ad5 hexon can be detected by immunohistochemistry late in the virus replication cycle. Figure 35A shows Hep3B xenograft tumors stained for Ad5 hexon 9 days following treatment intravenously with CN790. While positively stained cells were visible throughout the tumor, infected cells were predominantly located near the tumor periphery. No adenovirus was detected in vehicle treated tumors.

To estimate CN790 induced apoptosis, three CN790 treated and three control tumors were analyzed nine days after initial dosing (Figure 35B). Treated tumors had a 70% higher apoptotic index than control tumors. Because apoptosis is short-lived, even modest increases in the apoptotic index of a tumor may result in a significant antitumor effect. Bursch et al. (1992) *Trends Pharmacological Science* 13:245-251; and Bursch et al. (1990) *Carcinogenesis* 11:847-853. Apoptotic bodies were observed both adjacent to infected cells and uninfected areas, indicating that apoptosis may augment virus mediated killing. Thus, the immunohistochemical analysis of CN790 treated tumors suggests that

both virus replication-dependent cytolysis and apoptosis contribute to the CN790 antitumor effect.

Furthermore, CN790 caused plaques that were estimated to be 5 times larger than those lysed by CN733 on 293 cells 6 days after infection (Figure 31), indicating that CN790 possesses higher potency to induce apoptosis than CN733.

In summary, CN790 grows at least 100,000 fold less efficiently than the wild-type E1 viruses CN702 and CN802 in AFP⁻ cells (OVCAR-3 cells). Most importantly, intravenous administration of CN790 inhibited the growth of HCC xenografts and stabilized the concentration of serum tumor marker AFP to a greater extent than CN733. Immunohistochemical analysis of treated tumors confirmed that systemically administered CN790 productively infected the tumor mass and caused tumor killing. Furthermore, our analysis revealed that treated tumors had a higher apoptotic index than control tumors, suggesting that virus replication-dependent cytolysis and apoptosis were mechanisms of tumor destruction.

EXAMPLE 4

In vitro characterization of E3-containing adenoviral constructs comprising a CEA-TRE driving expression of E1A

To examine the cell-type specificity of CN802, CN799, and CN798, various cell lines were infected at a concentration of 2 PFU per cell with the constructs. The number of plaque-forming units was determined 48 hours post-infection, as described above. As shown in Figure 17, CN798, which contains an E3 region, plaqued efficiently on LoVo, and SW1463 cells, which synthesize CEA, but inefficiently on PA-1 and G361 cells, which do not. In contrast, CN799 and CN802 plaqued efficiently on all cell lines tested. Thus, CN798 appears to specific for CEA-expressing cell lines.

As another measure of cell specificity, LoVo, PA-1, and G361 cells were infected with CN802 or CN798, and viral DNA was measured. Equal amounts of cellular DNA were added to wells and quantitated by a slot blot assay. As shown in Figure 18, viral DNA was undetectable in PA-1 or G361 cells infected with CN798, while approximately 0.4 ng CN798 DNA was measured in infected LoVo cells. In contrast, CN802 viral DNA was detected in all four cell lines infected with this construct.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

5

CLAIMS

What is claimed is:

1. An adenovirus vector comprising
 - (a) an adenovirus gene under transcriptional control of a target cell-specific transcriptional regulatory element (TRE); and
 - (b) an E3 sequence.
2. The adenovirus vector of claim 1, wherein the adenovirus gene is essential for replication.
3. The adenovirus vector of claim 2, wherein the adenovirus gene is an early gene.
4. The adenovirus vector of claim 3, wherein the early gene is E1A.
5. The adenovirus vector of claim 3, wherein the early gene is E1B.
6. The adenovirus vector of claim 3, wherein the early gene is E2.
7. The adenovirus vector of claim 3, wherein the early gene is E4.
8. The adenovirus vector of claim 1, wherein the target cell-specific TRE is cell type specific.
9. The adenovirus vector of claim 8, wherein the cell type-specific TRE is prostate cell specific.
10. The adenovirus vector of claim 9, wherein the TRE is a PSA-TRE.
11. The adenovirus vector of claim 9, wherein the TRE is a PB-TRE.

12. The adenovirus vector of claim 8, wherein the cell type-specific TRE is liver cell specific.

13. The adenovirus vector of claim 12, wherein the TRE is an AFP-TRE.

5

14. The adenovirus vector of claim 8, wherein the cell type-specific TRE is breast cancer cell-specific.

15. The adenovirus vector of claim 14, wherein the TRE is a MUC1-TRE.

10

16. The adenovirus vector of claim 14, wherein the TRE is a CEA-TRE.

17. The adenovirus vector of claim 16, wherein the CEA-TRE comprises nucleotides -6072 to -3815 to -300 to +70 relative to the CEA gene transcription start site.

15

18. The adenovirus vector of claim 8, wherein the cell type-specific TRE is colon cancer cell-specific.

19. The adenovirus vector of claim 8, wherein the TRE is an hKLK2-TRE.

20

20. The adenovirus vector of claim 1, wherein the target cell-specific TRE is cell status specific.

21. The adenovirus vector of claim 20, wherein the cell status-specific TRE comprises a hypoxia-responsive element.

25

22. The adenovirus of claim 1 comprising an adenoviral gene under transcriptional control of a cell status-specific TRE and a cell type-specific TRE.

23. The adenovirus of claim 22, wherein the cell status-specific TRE comprises a hypoxia-responsive element (HRE) and the cell type-specific TRE is a prostate-specific antigen TRE (PSA-TRE).

5 24. The adenovirus vector of claim 23, wherein the HRE comprises the nucleotide sequence depicted in SEQ ID NO:9.

25. The adenovirus vector of claim 24, wherein the PSA-TRE comprises nucleotides -5322 to -3738 and -541 to +12 relative to the PSA gene transcription start site.

10

26. The adenovirus vector of claim 1, wherein the E3 sequence is under transcriptional control of a target cell-specific TRE.

27. An adenovirus vector comprising (a) an E3 sequence; and (b) a first adenovirus gene under transcriptional control of a first target cell-specific transcriptional response element (TRE) and a second gene under transcriptional control of a second target cell-specific TRE.

15

28. The adenovirus vector of claim 27, wherein the first adenovirus gene is essential for adenovirus replication.

20

29. The adenovirus vector of claim 27, wherein the gene essential for replication is an adenovirus early gene.

25 30. The adenovirus vector of claim 29, wherein the early gene is E1A.

31. The adenovirus vector of claim 29, wherein the early is E1B.

32. The adenovirus vector of claim 29, wherein the early gene is E2.

30

33. The adenovirus vector of claim 29, wherein the early gene is E4.

34. The adenovirus vector of claim 27, wherein the first and second genes are essential for adenovirus replication.

5 35. The adenovirus vector of claim 34, wherein the first and second genes are adenovirus early genes.

36. The adenovirus vector of claim 27, wherein the second gene is a transgene.

10 37. The adenovirus vector of claim 36, wherein said transgene is a cytotoxic gene.

38. The adenovirus vector of claim 27, wherein the first and second target cell-specific TREs are cell-type specific and functional in the same cell.

15 39. The adenovirus vector of claim 29, wherein the first and second target cell-specific TREs are prostate cell-specific.

40. The adenovirus vector of claim 39, wherein the first TRE is a *PB*-TRE.

20 41. The adenovirus vector of claim 39, wherein the second TRE is a *PSA*-TRE.

42. The adenovirus vector of claim 39, wherein the first TRE is a *PB*-TRE and the second TRE is a *PSA*-TRE.

25 43. The adenovirus vector of claim 42, wherein the *PB*-TRE comprises nucleotides -426 to +28 relative to the rat probasin gene transcription start site, and the *PSA*-TRE comprises nucleotides -5322 to -3738 and -541 to +12 relative to the *PSA* gene transcription start site.

30 44. The adenovirus vector of claim 27, wherein the first and second target cell-specific TREs are liver cell-specific.

45. The adenovirus vector of claim 44, wherein the first and second TREs are *AFP*-TREs.

5 46. The adenovirus vector of claim 45, wherein the *AFP*-TREs comprise nucleotides -3954 to -3335 and -174 to +29 relative to the *AFP* gene transcription start site.

47. The adenovirus vector of claim 46, wherein the first and second TREs are colon cancer cell-specific.

10

48. The adenovirus vector of claim 47, wherein the first and second TREs are *CEA*-TREs.

49. An isolated host cell comprising the adenovirus vector of claim 1.

15

50. An isolated host cell comprising the adenovirus vector of claim 27.

51. A composition comprising the adenovirus vector of claim 1.

20

52. A composition comprising the adenovirus vector of claim 27.

53. A method for conferring selective cytotoxicity on a cell which allows a target cell-specific TRE to function, comprising contacting said cell with an adenovirus vector of claim 2, wherein the adenovirus vector enters the cell.

25

54. A method according to claim 53, wherein cell is a mammalian cell.

55. A method according to claim 54, wherein the mammalian cell is a prostate cell.

30

56. A method according to claim 54, wherein the mammalian cell is a liver cell.

57. A method according to claim 54, wherein the mammalian cell is a breast cancer cell.

58. A method according to claim 55, wherein the mammalian cell is a colon cancer cell.

59. A method for propagating an adenovirus vector of claim 1, said method comprising combining an adenovirus vector of claim 1 with cells which allow function of the target cell-specific TRE, whereby said adenovirus is propagated.

60. A method according to claim 59, wherein the cells are mammalian cells.

61. A method for suppressing tumor growth comprising contacting a tumor cell with an adenovirus vector according to claim 2 such that the adenovirus vector is introduced into the tumor cell.

62. A method according to claim 61, wherein the tumor cell is a mammalian cell.

63. A method according to claim 62, wherein the mammalian cell is a prostate cell.

64. A method according to claim 61, wherein the mammalian cell is a liver cell.

65. A method according to claim 61, wherein the mammalian cell is a breast cancer cell.

66. A method according to claim 61, wherein the mammalian cell is a colon cancer cell.

67. A method for modifying the genotype of a target cell comprising contacting the target cell with the adenovirus vector according claim 1 such that the adenovirus vector is introduced into the target cell.

68. A replication competent adenovirus vector comprising an E3 sequence under transcriptional control of a target cell-specific TRE.

Region E3 of Adenovirus

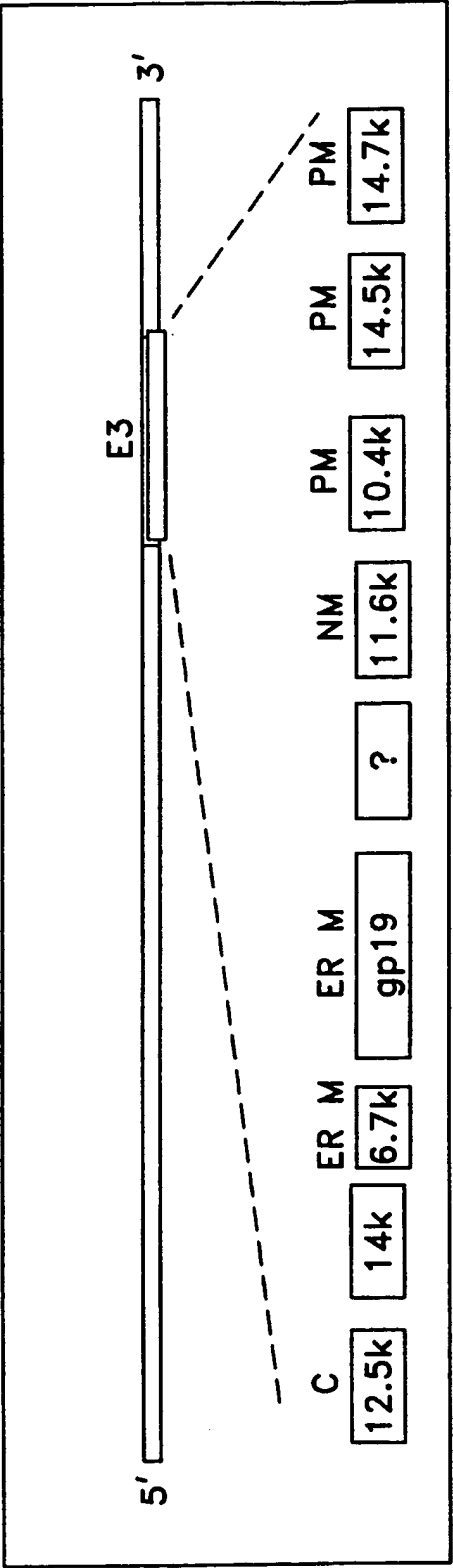


FIGURE 1

2/57

CN787=CN739+wtE3

		Killing	Specificity
CN702		100	1
CN706		30	100
CN739		30	10,000
CN787		~300	10,000

FIGURE 2

3/57

Specificity of CN787: 10,000:1

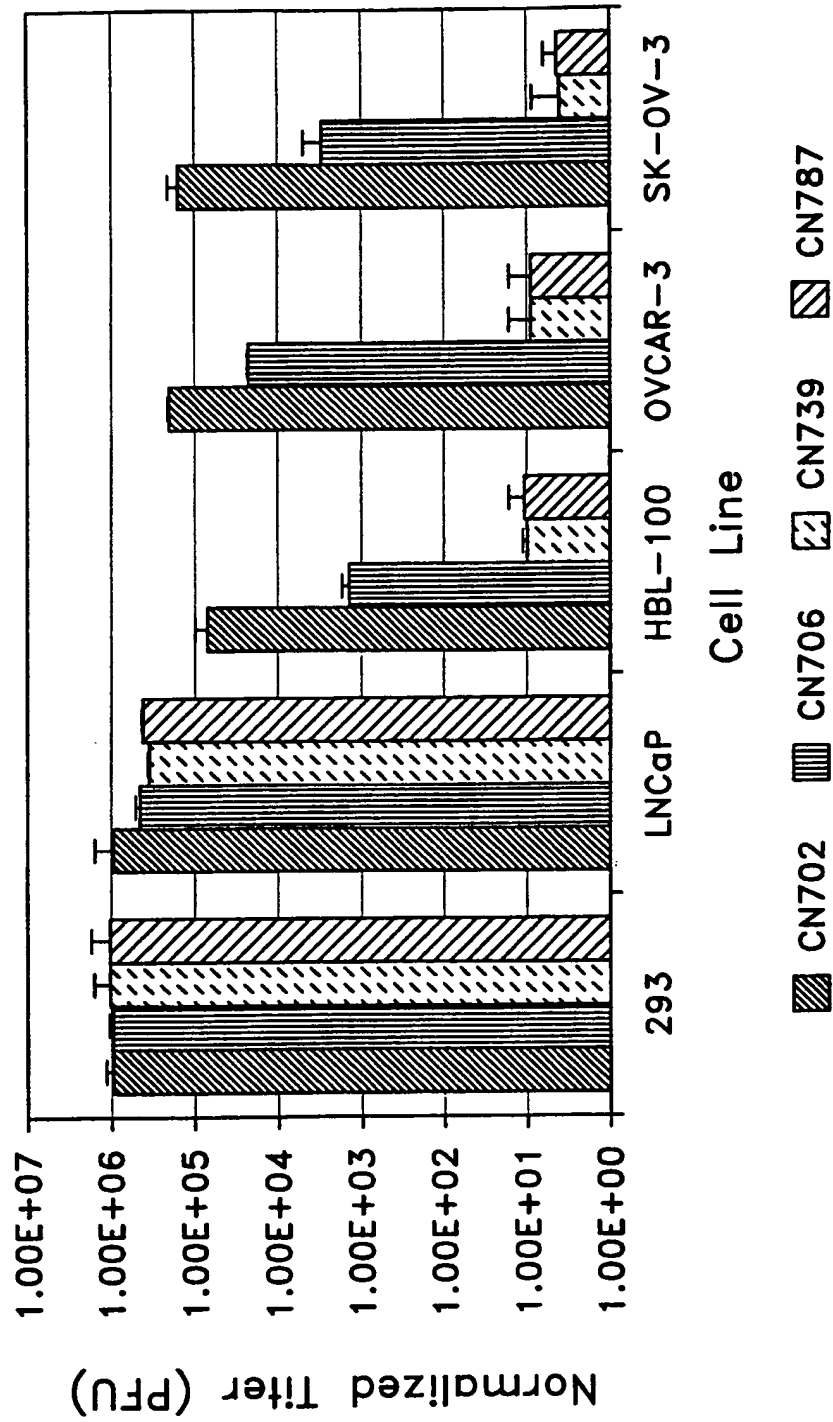


FIGURE 3

4/57

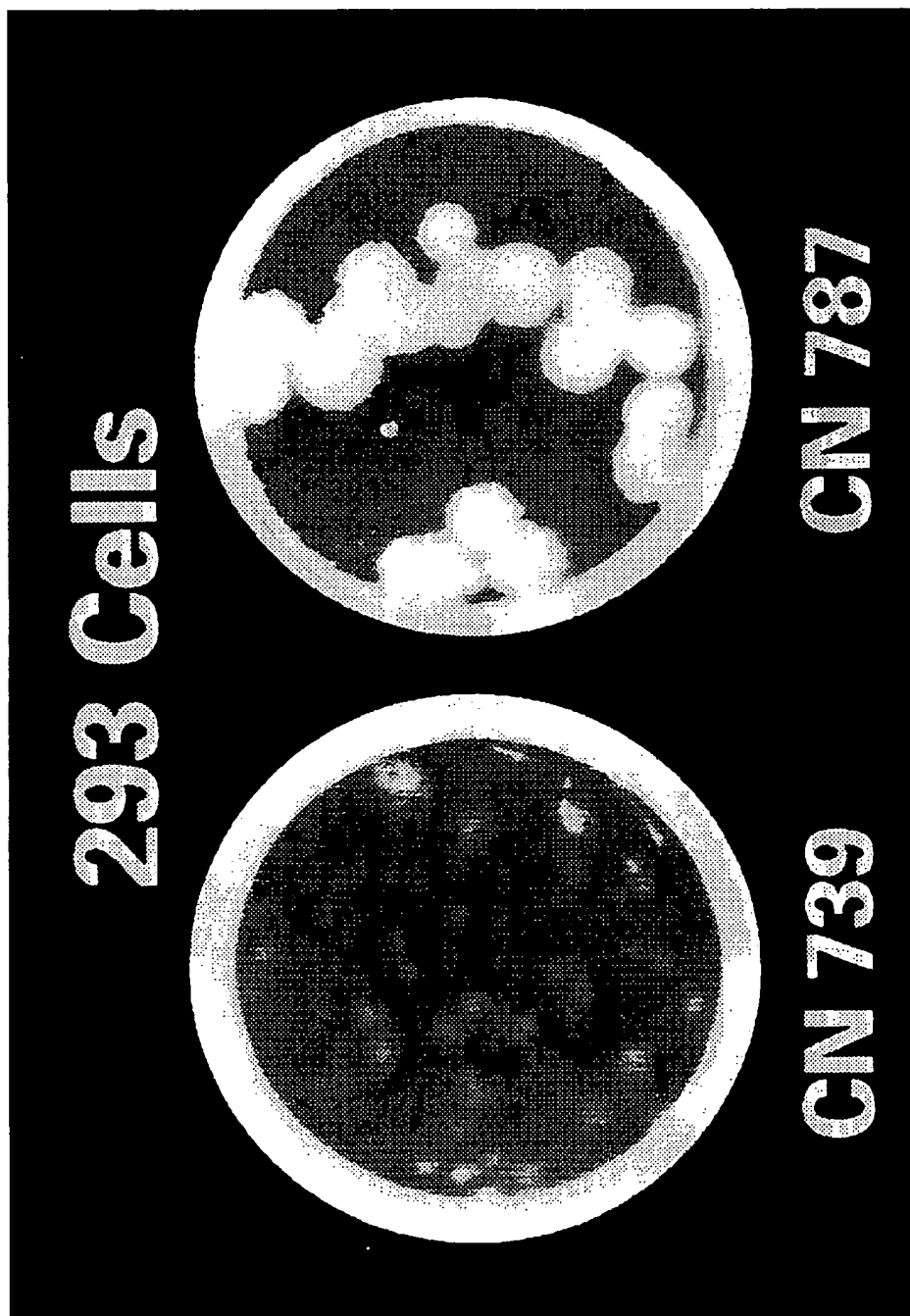


Figure 4

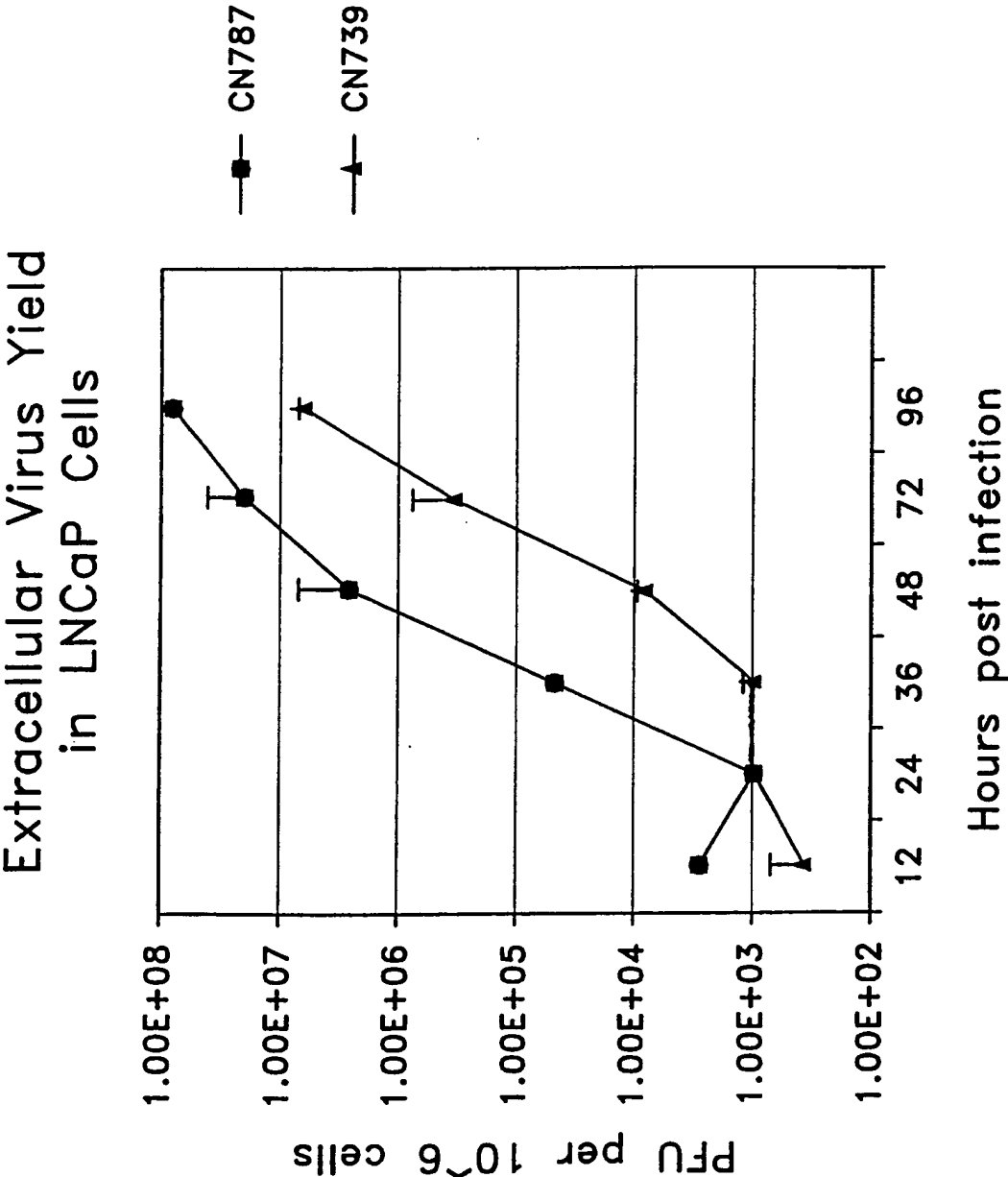


FIGURE 5

6/57

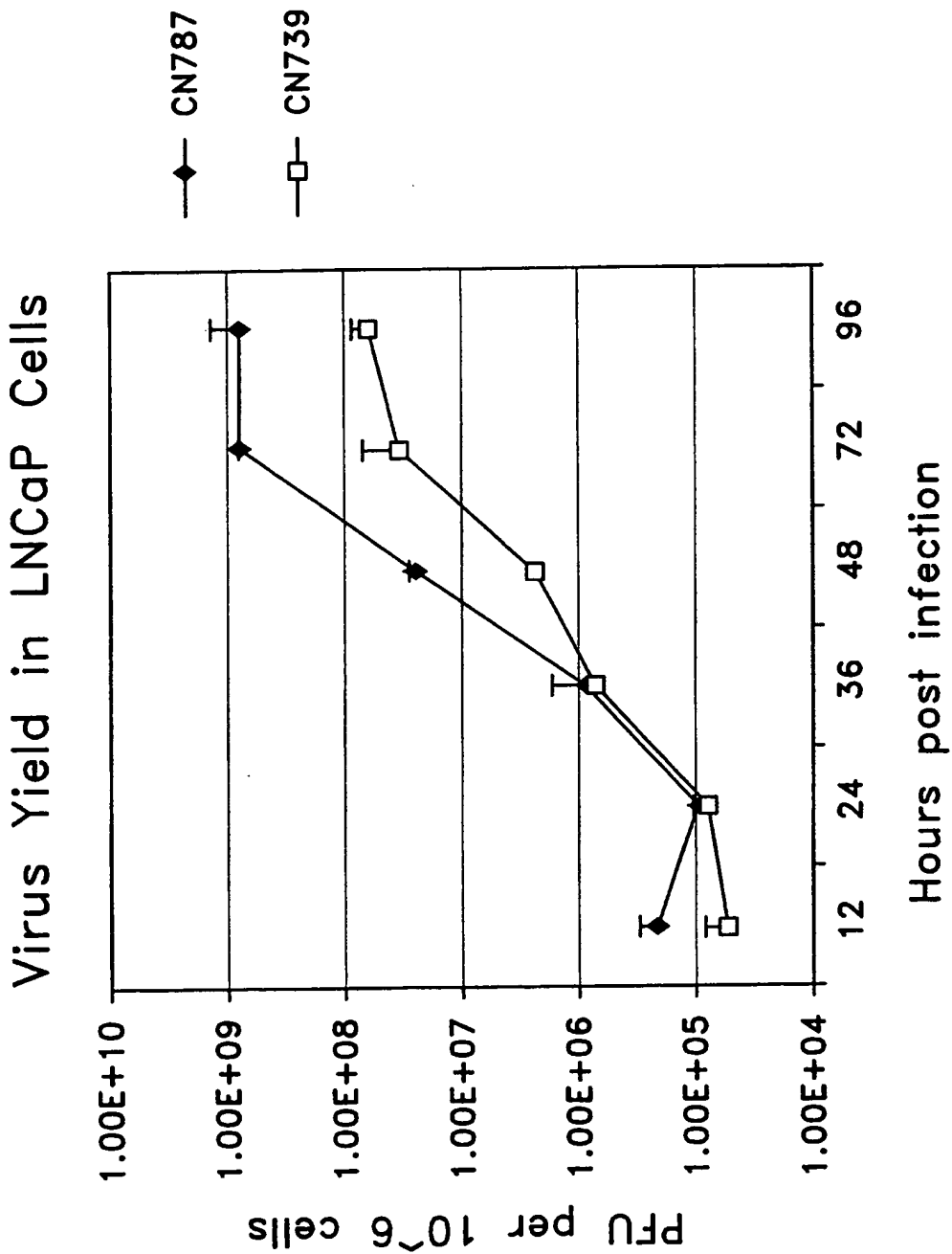


FIGURE 6

7/57

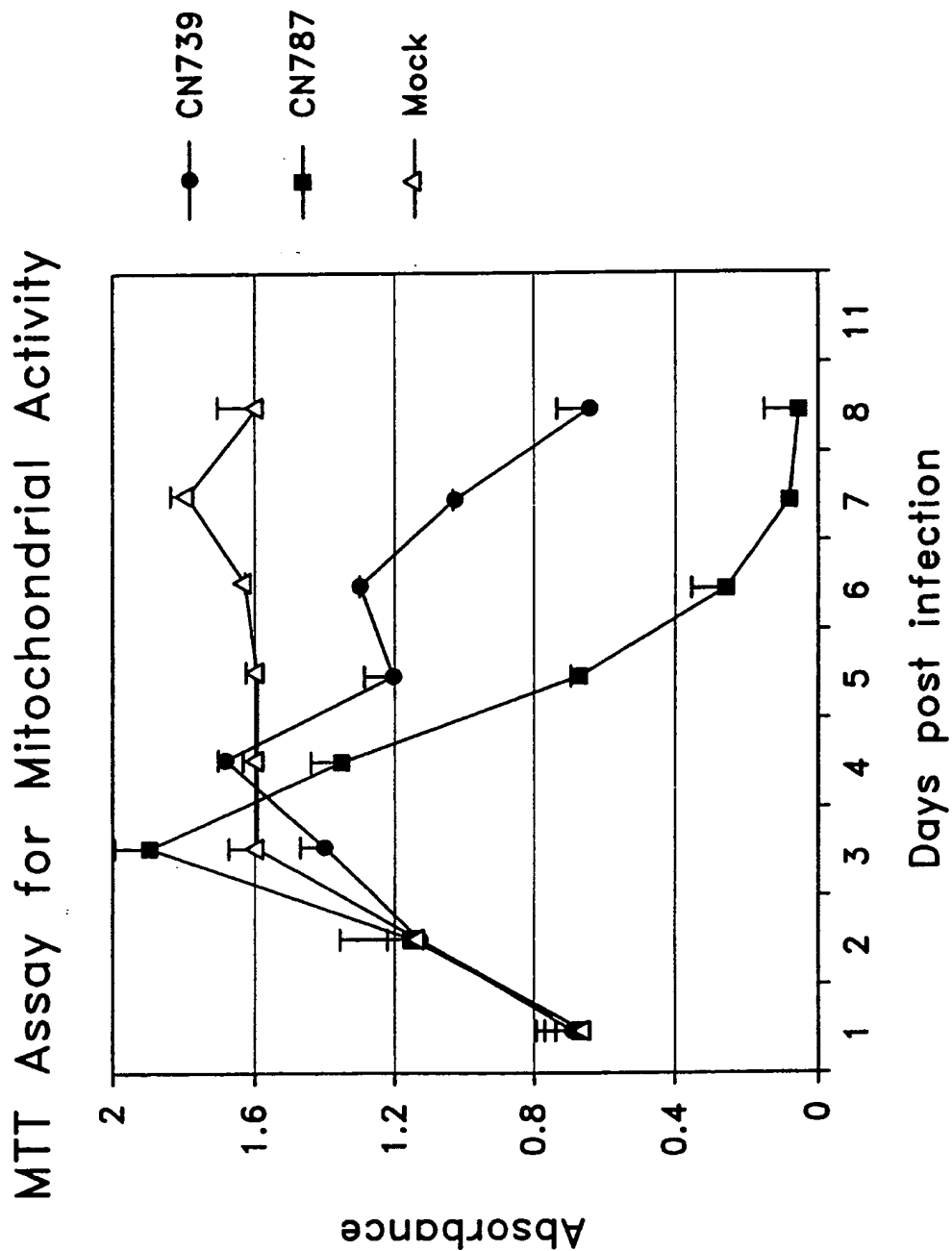


FIGURE 7

8/57

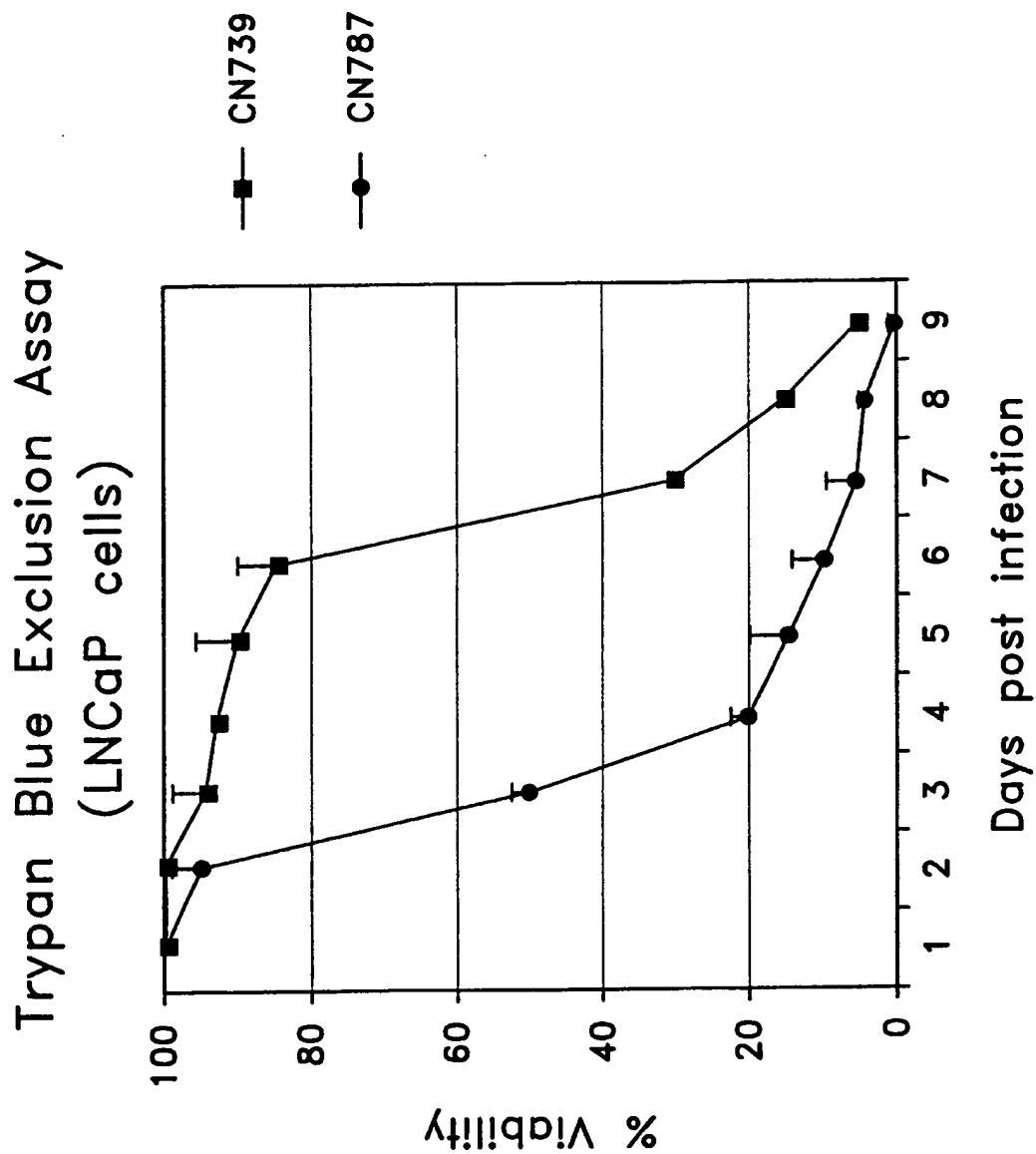


FIGURE 8

9/57

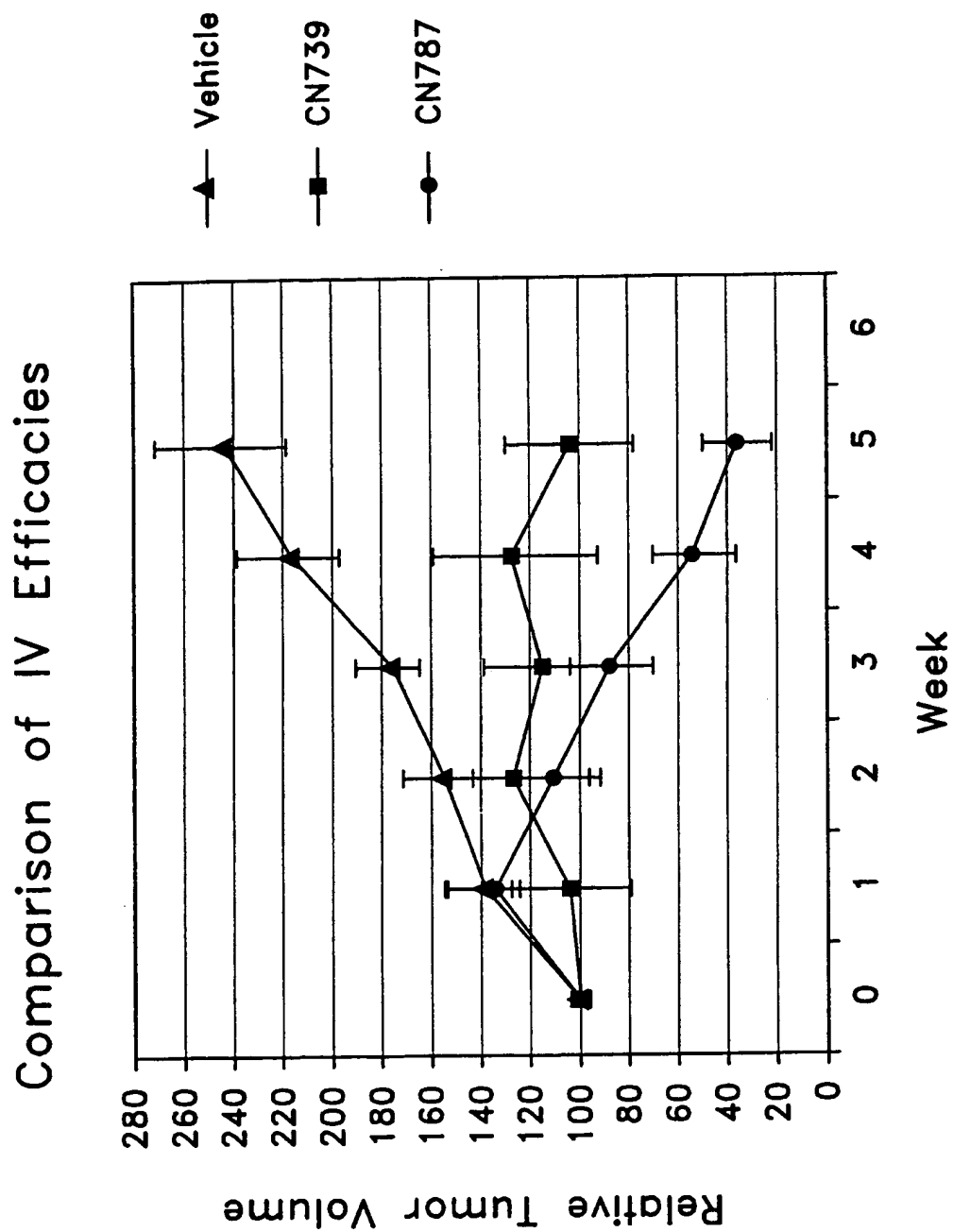


FIGURE 9

10/57

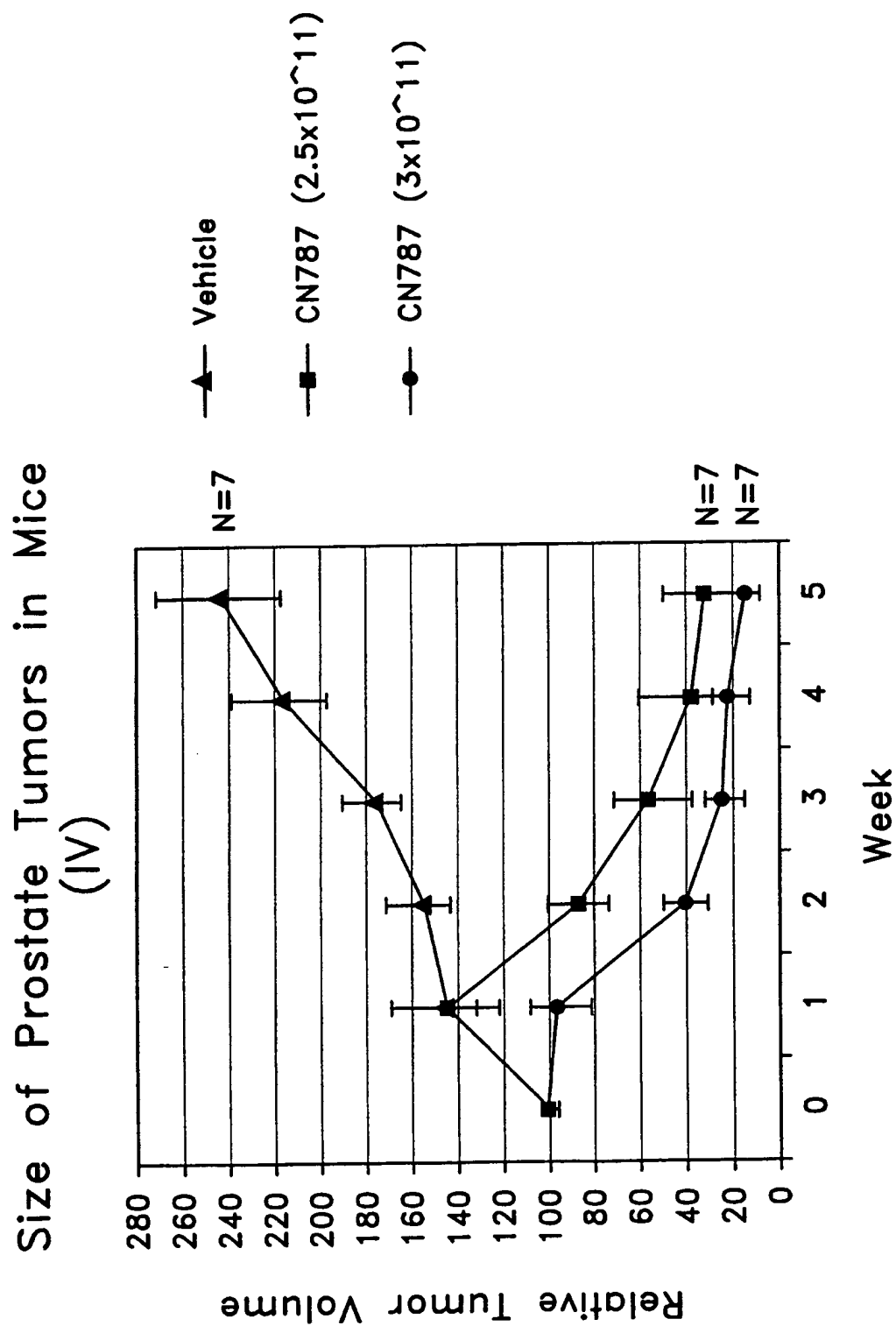


FIGURE 10

AFP E1A and E1B Adenovirus Constructs

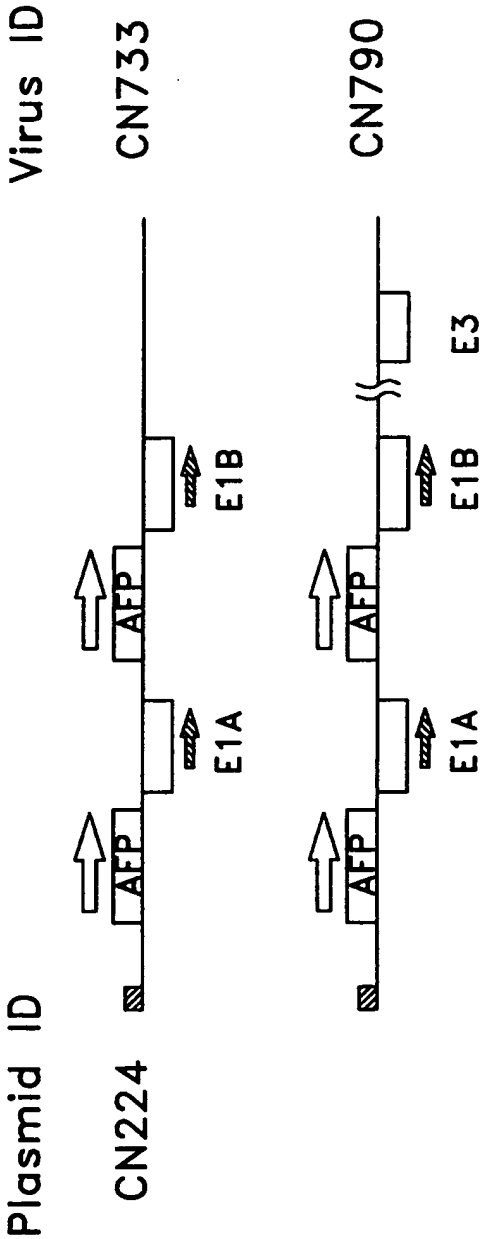


FIGURE 11

12/57

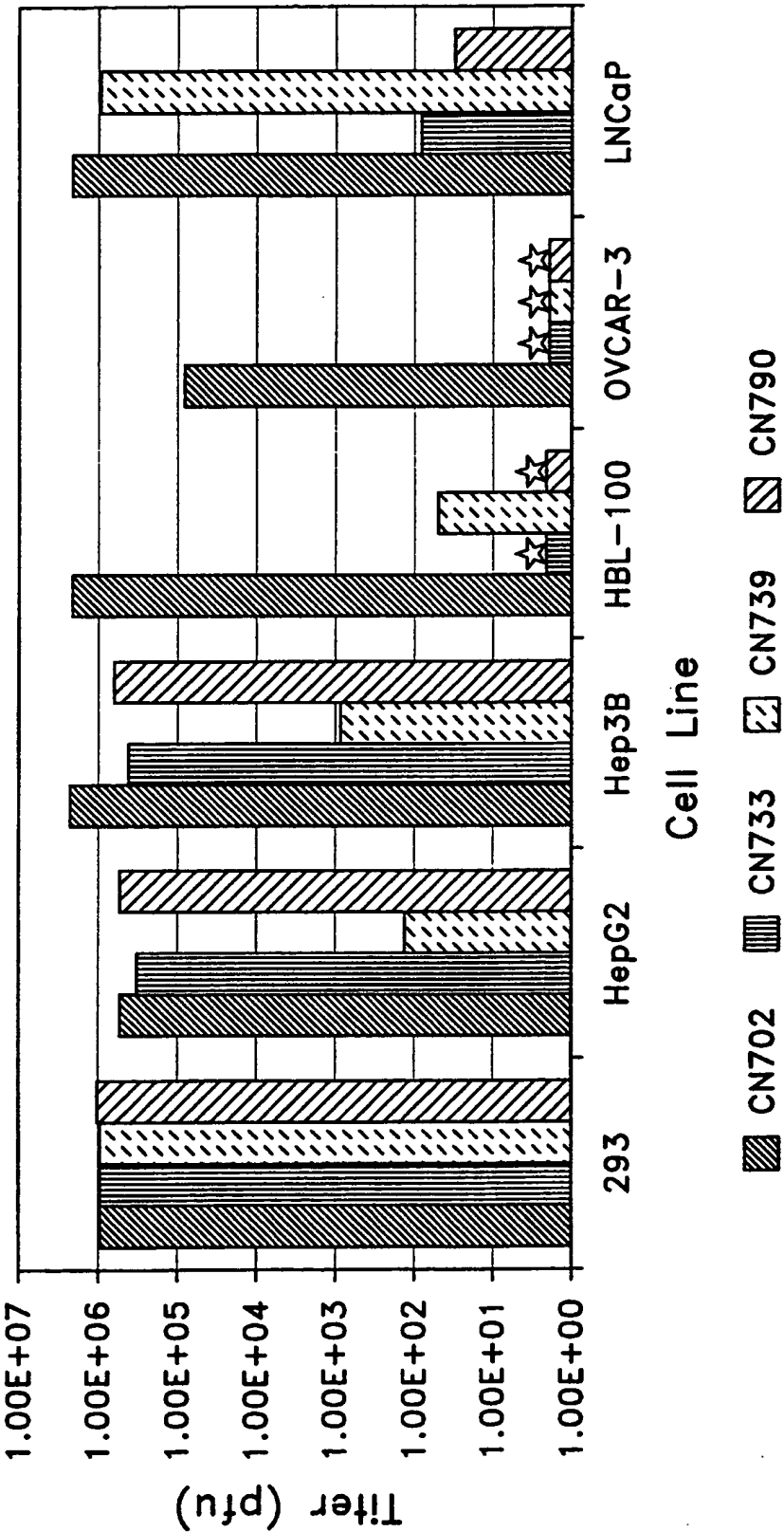


FIGURE 12

13/57

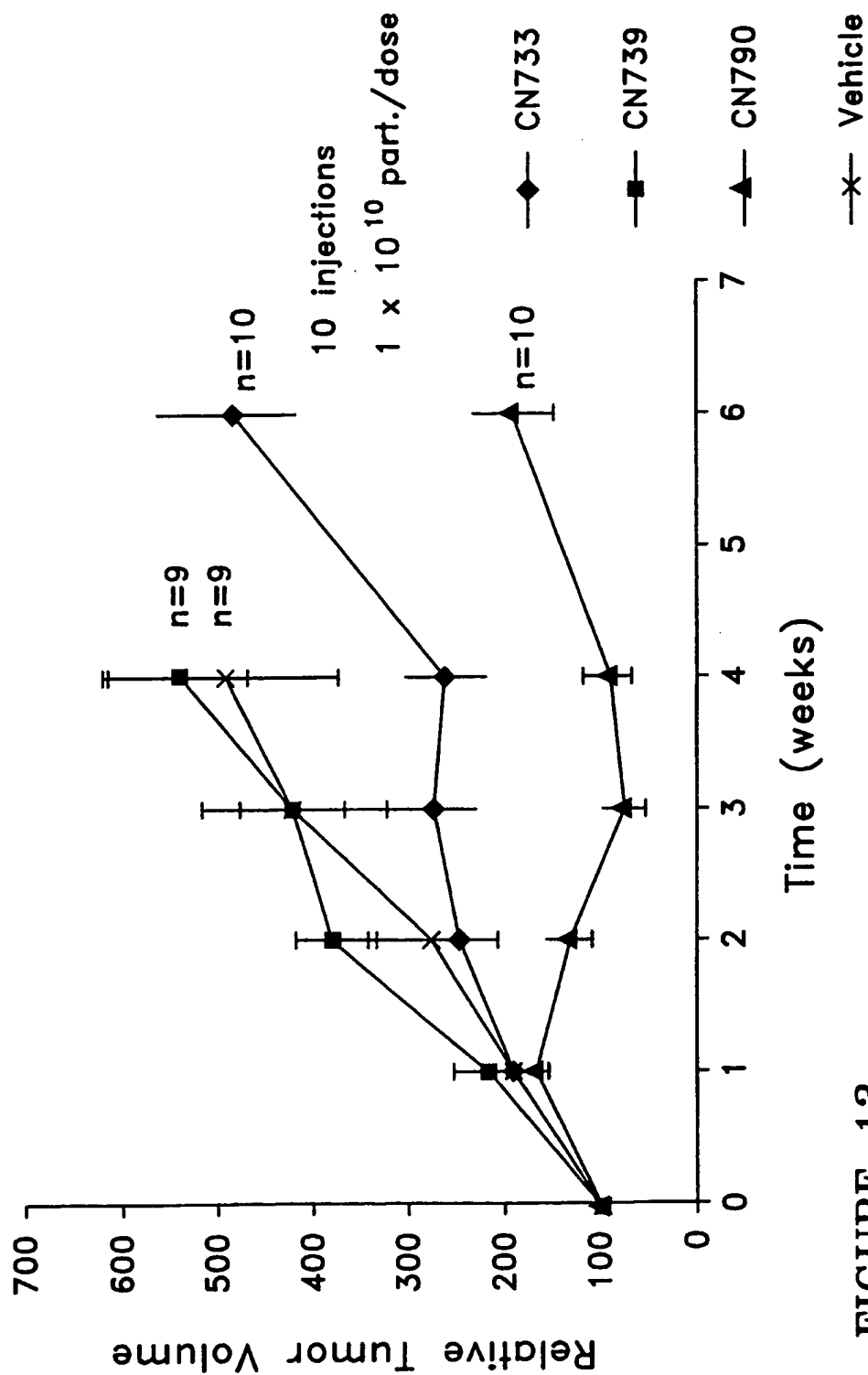


FIGURE 13

14/57

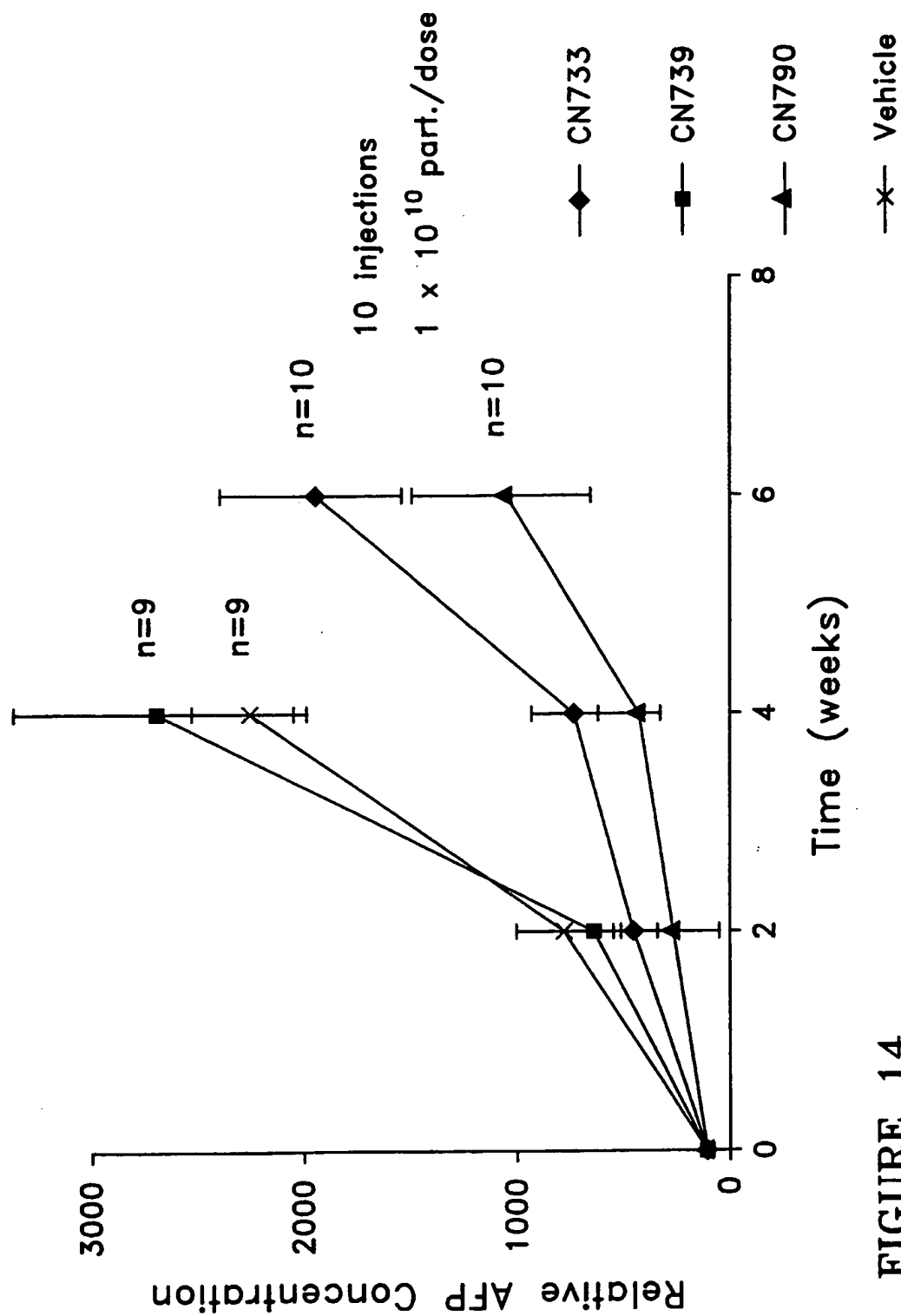
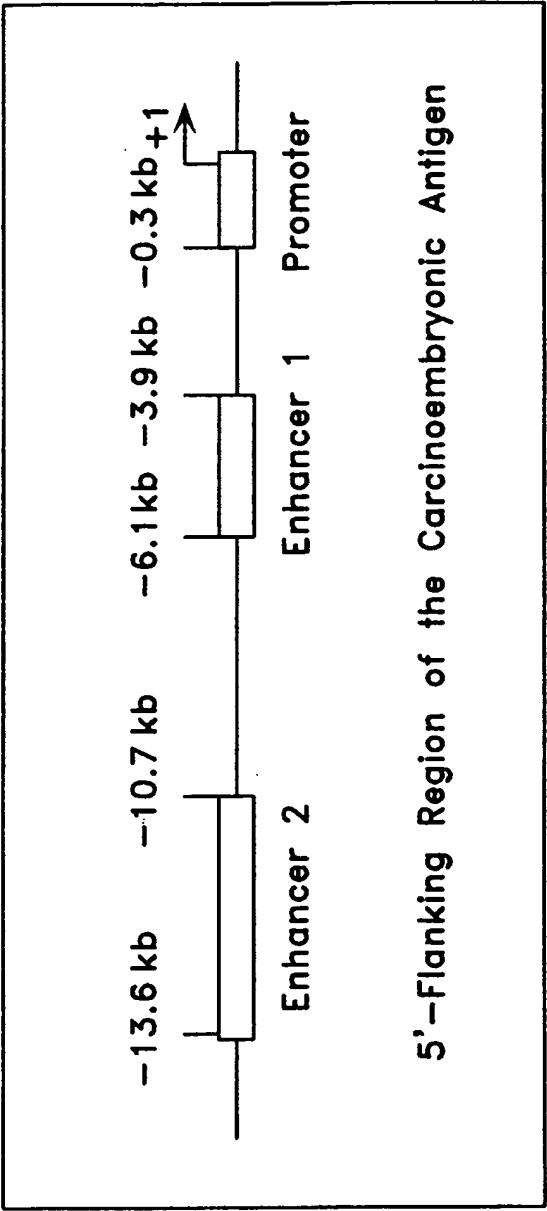


FIGURE 14

CEA Transcription Response Element
(CEA-TRE)



5'-Flanking Region of the Carcinoembryonic Antigen

FIGURE 15

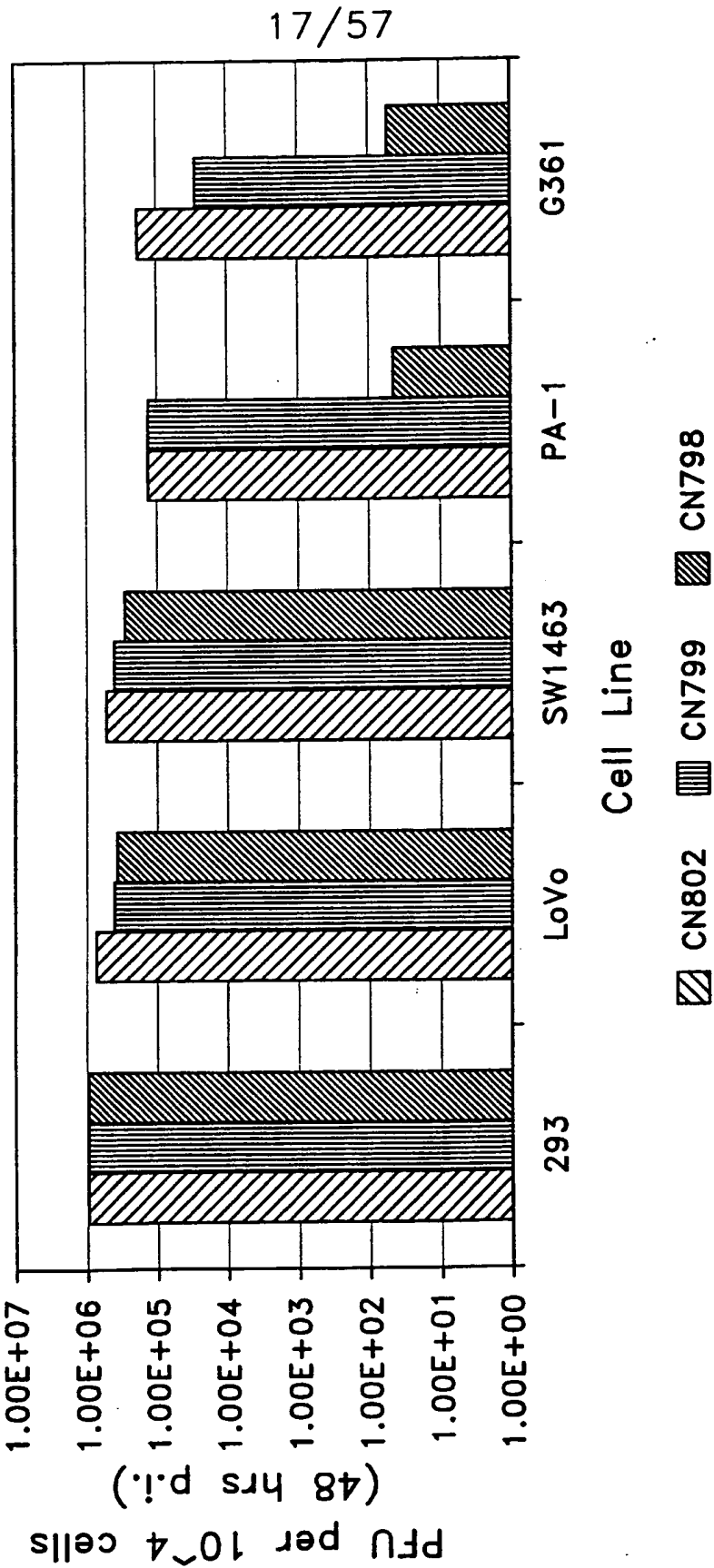


FIGURE 17

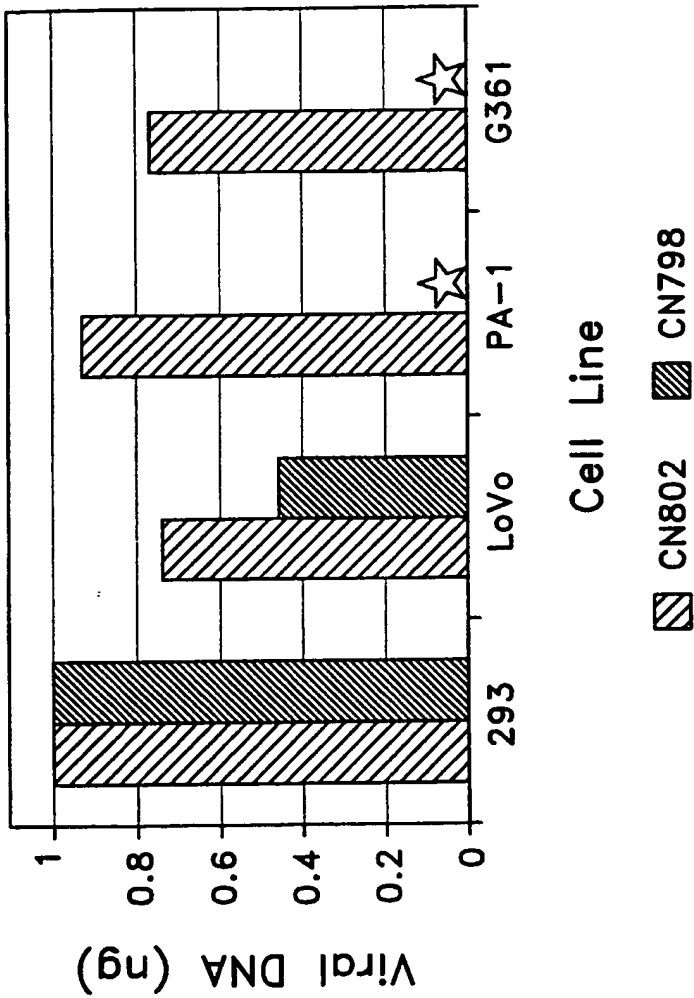


FIGURE 18

19/57



FIGURE 19

20/57

FIGURE 20

ccccgagg cagtgcac gaggctcagg gcgtgggt gaggcgcagagaccccg ggggtgcag gccggga

21/57

FIGURE 21

gggcccaaaa ttagcaagtg accacgtggg tctgaagcca gtggcctaag gaccacccctt 61
 gcagaaccgt ggtctccttg tcacagtcta ggcagcctctt ggcttagcct ctgtttctttt 121
 cataaccttt ctacgcgcct gctctgggoc agaccagtgt tgggaggagt cgctactgag 181
 ctctagatt ggcaggggag gcagatggag aaaaggagtg tgtgtggtca gcattggagc 241
 agaggcagca gtgggcaata gaggaaagtga gtaaatcctt gggaggggctc cctagaagtg 301
 atgtgttttc tttttttgtt ttagagacag gatctcgctc tgcgcccag gctgggtgtgc 361
 agtggcatga tcatagctca ctgcagcctc gacttctcgg gctcaagcaa tcccccacc 421
 tcagcctccc aagttagctgg gactacgggc acacgccacc atgcctggct aatttttgt 481
 ttttttgtag agatgggtct tcaccatgtt gatcaggctg gtctcgaact cctgggctca 541
 tgcgattccac cccgccagct gattacaggg attccgggtg tgagccaccg cgcccagacg 601
 ccacttcacg gtattgtaaa cgtctgttac ctttctgttc cctgtctac tggactgtga 661
 gctccttagg gccacgaatt gaggatgggg cacagagcaa gctctccaaa cgtttgttga 721
 atgagttagg gaatgaatga gtccaagcag atgctatacg ttggctgttg gagatttttg 781
 ctaaaatggg acttgacgga agcccgcag tccccctcg catttcagg caccgctctt 841
 cagcttgggc tctgggtgag cgggataggg ctgggtgcag ctgaggata atgcatggg 901
 tgaggcaagt tgaggatgga agaggtggct gatggctggg ctgtggaaact gatgacctg 961
 aaaagaagag gggacagtct ctggaaatct aagctgaggc tgttgggggc tacaggttga 1021
 ggtcacgtg cagaagagag gctctgttct gaacctgcac tatagaaagg tcagtgggat 1081
 gcgggagcgt cggggcgggg cggggcctat gtccccgtt cccacgcct ccagcagggg 1141
 acgccccggc tggggggcggg ggtcagacc ggcctgggt ccatccggac aaagcctgcg 1201
 cgccccccg cccgccattg gccgtaccgc cccgcgcgcg cgccccctcg 1261
 cgccgggtcc ggcgcgtaaa agccaatagg aaccgcgcgc gttgttcccc tccgggcgg 1321
 ggagcccaat tgtggcgcg ctcgggggct cgtggctctt tcgcgggcaaa aaggatttgg 1381
 cgcgtaaaag*tgcccgggac ttgacaggca gggcgggcgg gggcgggagc gggatcgagc 1441
 cctcgccgag gcctgcccgc atggggcccgc gccgcccgcg ccgcctgtca cccggggcgc 1501
 gcggggccgtg agcgtcatg

22/57

FIGURE 22A

aagcttctag ttttcttttc ccggtgacat cgtggaaagc actagcatct ctaagcaatg 60
atctgtgaca atattcacag tgtaatgcc a tccagggaac tcaactgagc cttgatgtcc 120
agagattttt gtgttttttt ctgagactga gtctcgtctt gtgccaggct ggagtgcagt 180
ggtgcaacct tggctcactg caagctccgc ctctggggtt cagccattc tcctgcctca 240
gcctcctgag tagctgggac tacaggcacc cgccaccacg cctggctaata ttttttgtat 300
ttttagtaga gatgggggtt cactgtgtta gccaggatgg tctcagtctc ctgacctcgt 360
gatctgcccc ccttggcctc ccaaagtgtt gggatgacag gcgtgagcca ccgcgcctgg 420
ccgatatcca gagatttttt ggggggctcc atcacacaga catgttgact gtcttcattg 480
ttgactttta gtatccagcc cctctagaaa tctagctgat atagtgtggc tcaaaacctt 540
cagcacaat cacaccgtta gactatctgg tgtggcccaa accttcaggt gaacaaaggg 600
actctaattt ggcaggatat tccaaagcat tagagatgac ctcttgcaaa gaaaaagaaa 660
tgaaaaagaa aaagaaagaa aggaazaaaa aaaaaaaaaa gagatgacct ctcaggctct 720
gaggggaaac gcctgaggtc tttgagcaag gtcagtcctc tgttgacag tctccctcac 780
agggtcattg tgacgatcaa atgtggtcac gtgtatgagg caccagcaca tgcctggctc 840
tggggagtgc cgtgtaagtg tatgcttgca ctgctgaatg cttgggatgt gtcagggatt 900
atcttcagca cttacagatg ctcatctcat cctcacagca tcactatggg atgggtatta 960
ctggcctcat ttgatggaga aagtggctgt ggctcagaaa ggggggacca ctgaccagg 1020
gacactctgg atgctgggga ctccagagac catgaccact caccaactgc agagaaatta 1080
attgtggcct gatgtccctg tcctggagag ggtggagggt gaccttcact aacctcctac 1140
cttgaccctc tcttttaggg ctctttctga cctccaccat ggtactagga cccattgta 1200
ttctgtacct tcttgactct atgaccccca ctgcccactg catccagctg ggtccctcc 1260
tatctctatt ccagctggc cagtgcagtc tcagtgccca cctgtttgtc agtaactctg 1320
aaggggctga ctttttactg acttgcaaac aaataagcta actttccaga gttttgtgaa 1380
tgctggcaga gtccatgaga ctctgagtc agaggcaaag gcttttactg ctcacagctt 1440
agcagacagc atgaggttca tgttcacatt agtacacctt gccccccca aatctttag 1500
ggtgaccaga gcagtctagg tggatgctgt gcagaagggg tttgtgccac tggtgagaaa 1560
cctgagatta ggaatcctca atcttatact gggacaactt gcaaacctgc tcagcctttg 1620
tctctgatga agatattatc ttcattgatc tggattgaaa acagacctac tctggaggaa 1680

23/57

FIGURE 22B

~~catattgtat~~cgattgtcct tgacagtaaa caaatctgtt gtaagagaca ttatctttat 1740
 tatctaggac agtaagcaag cctggatctg agagagatat catcttgcaa ggatgcctgc 1800
 ttacaaaca tccttgaaac aacaatccag aaaaaaaaaag gtgttgctgt ctttgctcag 1860
 aagacacaca gatacgtgac agaaccatgg agaattgcct cccaacgctg ttcagccaga 1920
 gccttccacc cttgtctgca ggacagtctc aacgttccac cattaaatac ttcttctatc 1980
 acatcctgct tctttatgcc taaccaaggt tctaggtccc gatcgactgt gtctggcagc 2040
 actccactgc caaaccaga ataaggcagc gctcaggatc ccgaaggggc atggctgggg 2100
 atcagaactt ctgggtttga gtgaggagtg ggtccaccct cttgaatttc aaaggaggaa 2160
 gaggctggat gtgaaggtag tgggggaggg aaagtgtcag ttccgaactc ttaggtcaat 2220
 gagggaggag actggtaagg tccagctcc cgaggtagt atgtgggaat ggcctaagaa 2280
 tctcatatcc tcaggaagaa ggtgctggaa tctgagggg tagagtctc ggtatatttg 2340
 tggcttaagg ctctttggcc cctgaaggca gaggtggaa ccattaggtc cagggtttgg 2400
 ggtgatagta atgggatctc ttgattcctc aagagtctga ggatcgaggg ttgccattc 2460
 ttccatcttg ccacctaatc cttactccac ttgagggat caccagccct tctagctcca 2520
 tgaagggtccc ctgggcaagc acaatctgag catgaaagat gcccagagg ccttgggtgt 2580
 catccactca tcatccagca tcacactctg aggggtgtggc cagcaccatg acgtcatgtt 2640
 gctgtgacta tccctgcagc gtgccctccc agccacctgc caaccgtaga gctgcccatc 2700
 ctctctggtt gggagtggcc tgcattgtgc caggctgagg cctagtgtca gacagggagc 2760
 ctggaatcat agggatccag gactcaaaag tgctagagaa tggccatatg tcaccatcca 2820
 tgaaatctca agggcttctg ggtggagggc acagggacct gaacttatgg tttcccaagt 2880
 ctattgctct cccaagttag tctccagat acgaggcact gtgccagcat cagccttatc 2940
 tccaccacat cttgtaaaag gactaccag ggcctgatg aacaccatgg tgtgtacagg 3000
 agtagggggt ggaggcacgg actcctgtga ggtcacagcc aaggagcat catcatgggt 3060
 ggggaggagg caatggacag gcttgagaac ggggatgtgg ttgtatttgg ttttctttgg 3120
 ttagataaag tcttgggtat aggatgaga gtggagtatg aagaccagtt aggatggagg 3180
 atcagattgg acttgggtta gataaagtgc tgggtatagg attgagagtg gagtatgaag 3240
 accagttagg atggaggatc agattggagt tgggttagag atggggtaaa attgtgctcc 3300
 ggatgagttt gggattgaca ctgtgaggt ggtttgggat ggcatggctt tgggatggaa 3360

24/57

FIGURE 22C

atagatttgt tttgatgttg gctcagacat ccttggggat tgaactgggg atgaagctgg 3420
 gtttgatttt ggaggtagaa gacgtggaag tagctgtcag atttgacagt ggccatgagt 3480
 tttgtttgat ggggaatcaa acaatggggg aagacataag gggtggcttg ttaggttaag 3540
 ttgcgttggg ttgatggggg cggggctgtg tataatgcag ttggattggg ttgtattaaa 3600
 ttgggttggg tcaggttttg gttgaggatg agttgaggat atgcttgggg acaccggatc 3660
 catgagggttc tcaactggagt ggagacaaac ttcctttcca ggatgaatcc aggggaagcct 3720
 taattcacgt gtaggggagg tcaggccact ggctaagtat atccttcac tccagctcta 3780
 agatggtcct aaattgtgat tatctatata cacttctgtc tccctcactg tgcttgagg 3840
 ttacctgac actcaactag aaacagggga agattttatc aaattccttt tttttttttt 3900
 ttttttttga gacagagtct cactctgttg cccaggctgg agtgcagtgg cgcagtctcg 3960
 gctcactgca acctctgcct cccagggtca agtgattctc ctgcctcagc ctctgagtt 4020
 gctgggatta caggcatgca gcaccatgcc cagctaattt ttgtattttt agtagagatg 4080
 gggtttcacc aatgtttgcc aggcctggcct cgaactcctg acctgggtgat ccacctgcct 4140
 cagcctccca aagtgtctggg attacaggcg tcagccaccg cggccagcca cttttgtcaa 4200
 attcttgaga cacagctcgg gctggatcaa gtgagctact ctggttttat tgaacagctg 4260
 aaataaccaa ctttttgaa attgatgaa tcttacggag ttaacagtgg aggtaccagg 4320
 gctcttaaga gttcccgatt ctcttctgag actacaaatt gtgattttgc atgccacctt 4380
 aatctttttt tttttttttt taaatcgagg tttcagtctc attctatttc ccaggctgga 4440
 gttcaatagc gtgatcacag ctcaactgtg ccttgaacfc ctggccttaa gagattctcc 4500
 tgcttcggtc tccaatagc taagactaca gtagtccacc accatatcca gataattttt 4560
 aaattttttg gggggccggg cacagtggct cacgcctgta atcccaacac catgggaggg 4620
 tgagatgggt ggatcacgag gtcaggagtt tgagaccagc ctgaccaaca tggtgaaact 4680
 ctgtctctac taaaaaaaaa aaaaatagaa aaattagccg ggcgtggtgg cacacggcac 4740
 ctgtaatccc agctactgag gaggtgagg caggagaatc acttgaacct agaaggcaga 4800
 ggttgcaatg agccgagatt gcgccactgc actccagcct gggtgacaga gtgagactct 4860
 gtctcaaaaa aaaaaaattt tttttttttt ttgtagaga tggatcttgc tttgtttctc 4920
 tggttggcct tgaactcctg gttcaagtg atcctcctac cttggcctcg gaaagtgttg 4980
 ggattacagg cgtgagccac catgactgac ctgtcgttaa tcttgaggta cataaacctg 5040
 gctcctaaag gctaaaggct aaatatttgt tggagaaggg gcattggatt ttgcatgagg 5100

25/57

FIGURE 22D

atgattctg~~c~~cctgggaggg caggtcagca ggcattctctg ttgcacagat agagtgtaca 5160
ggctctggaga acaaggagtg ggggggttatt ggaattccac attgtttgct gcacgttgga 5220
ttttgaaatg ctagggaact ttgggagact catatttctg ggctagagga tctgtggacc 5280
acaagatctt tttatgatga cagtagcaat gtatctgtgg agctggattc tgggttggga 5340
gtgcaaggaa aagaatgtac taaatgccaa gacatctatt tcaggagcat gaggaataaa 5400
agttctagtt tctggtctca gagtggtgca gggatcaggg agtctcacia tctcctgagt 5460
gctggtgtct tagggcacac tgggtcttgg agtgcaaagg atctaggcac gtgaggcttt 5520
gtatgaagaa tcggggatcg taccacccc ctgtttctgt ttcattcttg gcatgtctcc 5580
tctgcctttg tcccctagat gaagtctcca tgagctacaa gggcctgggt catccagggt 5640
gatctagtaa ttgcagaaca gcaagtgcta gctctccctc cccttcacac gctctgggtg 5700
tgaggagggg ttgtccagcc tccagcagca tggggagggc cttggtcagc ctctgggtgc 5760
cagcagggca ggggcggagt cctggggaat gaaggtttta tagggctcct gggggaggct 5820
ccccagcccc aagctt 5836

26/57

FIGURE 23A

aagctttttta gtgcttttaga cagtgaagctg gtctgtctaa cccaagtac ctgggtccca	60
tactcagccc cagaagtga ggggtgaagct ggggtggagcc aaaccaggca agcctaccct	120
cagggtctccc agtggcctga gaaccattgg acccaggacc cattacttct agggtaagga	180
aggtacaaac accagatcca accatggtct ggggggacag ctgtcaaag cctazaaata	240
tacctgggag aggagcaggc aaactatcac tgccccaggt tctctgaaca gaaacagagg	300
ggcaacccaa agtccaaatc caggtgagca ggtgcaccaa atgccagag atatgacgag	360
gcaagaagtg aaggaaccac ccctgcatca aatgttttgc atgggaagga gaagggggtt	420
gctcatgttc ccaatccagg agaatgcatt tgggatctgc cttcttctca ctccttggtt	480
agcaagacta agcaaccagg actctggatt tggggaaaga cgtttatttg tggaggccag	540
tgatgacaat cccacgaggg cctaggtgaa gagggcagga aggctcgaga cactggggac	600
tgagtgaaaa ccacacccat gatctgcacc acccatggat gctccttcat tgctcacctt	660
tctgttgata tcagatggcc ccattttctg taccttcaca gaaggacaca ggctagggtc	720
tgtgcatggc cttcatcccc ggggccatgt gaggacagca ggtgggaaag atcatgggtc	780
ctcctgggtc ctgcagggcc agaacattca tcaccatac tgacctccta gatgggaatg	840
gcttccttgg ggctgggcca acggggcctg ggcaggggag aaaggacgtc aggggacagg	900
gaggaagggg catcgagacc cagcctggaa ggttcttgtc tctgaccatc caggatttac	960
ttccttgcac ctacctttgg tcattttccc tcagcaatga ccagctctgc ttctgatct	1020
cagcctccca ccctggacac agcaccacag tccctggccc ggctgcatcc acccaatacc	1080
ctgataaccc aggacccatt acttctaggg taaggagggt ccaggagaca gaagctgagg	1140
aaaggtctga agaagtcaca tctgtcctgg ccagagggga aaaaccatca gatgctgaac	1200
caggagaatg ttgaccacag aaagggaccg aggaccaag aaaggagtca gaccaccagg	1260
gtttgcctga gaggaaggat caaggccccg agggaaagca gggctggctg catgtgcagg	1320
aaactgggtg ggcataatgt tcttagatc tccctgaatt cagtgtccct gccatggcca	1380
gactctctac tcaggcctgg acatgctgaa ataggacaat ggccttgtcc tctctcccca	1440
ccatttgcca agagacataa aggacattcc aggacatgcc ttcctgggag gtccaggttc	1500
tctgtctcac acctcaggga ctgtagttac tgcacagcc atggtaggtg ctgatctcac	1560
ccagcctgtc caggcccttc cactctccac tttgtgacca tgtccaggac caccctcag	1620
atcctgagcc tgcaaatacc cccttgctgg gtgggtggat tcagtaaaca gtgagtcct	1680

27/57

FIGURE 23B

atccagcecc-gagagccacc tctgtcacct tctgtctggg catcatccca ccttcacaag	1740
cactaaagag catggggaga cctggctagc tgggtttctg catcacaaag aaaataatcc	1800
cccaggttcg gattcccagg gctctgtatg tggagctgac agacctgagg ccaggagata	1860
gcagaggtca gccctagggg ggggtgggtca tccacccagg ggacaggggt gcaccagcct	1920
tgctactgaa agggcctccc caggacagcg ccatcagccc tgcctgagag ctttgctaaa	1980
cagcagtcag aggaggccat ggcagtggct gagctcctgc tccaggcccc aacagaccag	2040
accaacagca caatgcagtc ctcccccaac gtcacaggtc accaaaggga aactgaggtg	2100
ctacctaacc ttagagccat caggggagat aacagcccaa tttcccaaac aggccagttt	2160
caatcccatg acaatgacct ctctgctctc attcttccca aaataggacg ctgattctcc	2220
cccaccatgg atttctccct tgtcccggga gccttttctg cccctatga tctgggcact	2280
cctgacacac acctcctctc tggtgacata tcagggtccc tcaactgtca gcagtccaga	2340
aaggacagaa ccttggaacg cgcccatctc agcttcaccc ttctctcttc acagggttca	2400
gggcaaagaa taaatggcag aggccagtga gccagagat ggtgacaggc agtgaccag	2460
gggcagatgc ctggagcagg agctggcggg gccacaggga gaaggatg caggaaggga	2520
aaccagaaa tgggcaggaa aggaggacac aggctctgtg gggctgcagc ccagggttgg	2580
actatgagtg tgaagccatc tcagcaagta aggccaggtc ccatgaacaa gagtgggagc	2640
acgtggcttc ctgctctgta tatgggggtg gggattccat gccccataga accagatggc	2700
cggggttcag atggagaagg agcaggacag gggatcccca ggataggagg accccagtgt	2760
ccccccag gcaggtgact gatgaatggg catgcagggt cctcctgggc tgggctctcc	2820
ctttgtccct caggattcct tgaaggaaca tccggaagcc gaccacatct acctggtggg	2880
ttctggggag tccatgtaa gccaggagct tgtgttgcta ggaggggtca tggcatgtgc	2940
tgggggcacc aaagagagaa acctgagggc aggcaggacc tggctctgagg aggcattggg	3000
gccagatgg ggagatggat gtcaggaaag gctgccccat caggaggggt gatagcaatg	3060
gggggtctgt gggagtgggc acgtgggatt ccctgggctc tgccaagttc cttcccatag	3120
tcacaacctg gggacactgc ccatgaaggg gcgccttgc ccagccagat gctgctgggt	3180
ctgccccatcc actacctct ctgctccagc cactctgggt ctttctccag atgccctgga	3240
cagccctggc ctgggcctgt cccctgagag gtgttgggag aagctgagtc tctggggaca	3300
ctctcatcag agtctgaaag gcacatcagg aaacatccct ggtctccagg actaggcaat	3360

FIGURE 23C

gaggaaaggg cccagctcc tccctttgcc actgagaggg tcgaccctgg gtggccacag	3420
tgacttctgc gtctgtccca gtcaccctga aaccacaaca aaaccccagc cccagaccct	3480
gcaggtacaa tacatgtggg gacagtctgt acccagggga agccagttct ctcttcctag	3540
gagaccgggc ctcaaggctg tgcccggggc aggcgggggc agcacgtgcc tgtccttgag	3600
aactcgggac cttaagggtc tctgctctgt gaggcacagc aaggatcctt ctgtccagag	3660
atgaaagcag ctctgcccc tcctctgacc tcttctctct tcccaaattct caaccaacaa	3720
ataggtgttt caaatctcat catcaaattc tcatccatcc acatgagaaa gcttaaaacc	3780
caatggattg acaacatcaa gagttggaac aagtggacat ggagatgtta cttgtggaaa	3840
tttagatgtg ttcagctatc gggcaggaga atctgtgtca aattccagca tggttcagaa	3900
gaatcaaaaa gtgtcacagt ccaaattgtc aacagtgcag gggataaaac tgtggtgcat	3960
tcaaactgag ggatattttg gaacatgaga aaggaaggga ttgctgctgc acagaacatg	4020
gatgatctca cacatagagt tgaaagaaag gagtcaatcg cagaatagaa aatgatcact	4080
aattccacct ctataaagtt tccaagagga aaacccaatt ctgctgctag agatcagaat	4140
ggaggtgacc tgtgccttgc aatggctgtg agggtcacgg gagtgtcact tagtgcaggc	4200
aatgtgccgt atcttaattc gggcagggct ttcatgagca cataggaatg cagacattac	4260
tgctgtgttc attttacttc accggaaaag aagaataaaa tcagccgggc gcggtggctc	4320
acgcctgtaa tcccagcact ttagaaggct gaggtgggca gattacttga ggtcaggagt	4380
tcaagaccac cctggccaat atggtgaaac cccggctcta ctaaaaatac aaaaattagc	4440
tgggcatggt ggtgcgcgcc tgtaatccca gctactcggg aggctgaggc tggacaattg	4500
cttggaacca ggaagcagag gttgcagtga gccaagattg tgccactgca ctccagcttg	4560
ggcaacagag ccagactctg taaaaaaaaa aaaaaaaaaa aaaaaaagaa agaaagaaaa	4620
agaaaagaaa gtataaaatc tctttgggtt aacaaaaaaaa gatccacaaa acaaacacca	4680
gctcttatca aacttacaca actctgccag agaacaggaa acacaaatac tcattaactc	4740
acttttgtgg caataaaacc ttcattgtca aaggagacca ggacacaatg aggaagttaa	4800
actgcaggcc ctacttgggt gcagagaggg aaaatccaca aataaaacat taccagaagg	4860
agctaagatt tactgcattg agttcattcc ccaggatgac aagggtgattt taacacctga	4920
aatcaatca ttgcctttac tacatagaca gattagctag aaaaaatta caactagcag	4980
aacagaagca atttggcctt cctaaaattc cacatcatat catcatgatg gagacagtgc	5040
agacgccaat gacaataaaa agagggacct ccgtcaccgc gtaaactatg ccacacagct	5100

29/57

FIGURE 23D

ccagcaagca- eccgtcttcc cagtgaatca ctgtaacctc ccctttaatc agccccaggg	5160
aaggctgcct gcgatggcca cacaggctcc aaccggtggg cctcaacctc ccgcagaggg	5220
tctcctttgg ccaccccatg gggagagcat gaggacaggg cagagccctc tgatgccac	5280
acatggcagg agctgacgcc agagccatgg gggctggaga gcagagctgc tggggtcaga	5340
gcttcctgag gacaccaggg cctaagggaa ggcagctccc tggatggggg caaccaggct	5400
ccgggctcca acctcagagc ccgcattggga ggagccagca ctctaggcct ttcctagggt	5460
gactctgagg ggaccctgac acgacaggat cgctgaatgc acccgagatg aagggggccac	5520
cacgggaccc tgctctcgtg gcagatcagg agagagtggg acaccatgcc agggcccat	5580
ggcatggctg cgactgaccc aggcactcc cctgcatgca tcagcctcgg taagtccat	5640
gaccaagccc aggaccaatg tggaaggaag gaaacagcat cccctttagt gatggaaccc	5700
aaggtcagtg caaagagagg ccatgagcag ttaggaaggg tggccaacc tacagcaca	5760
accatcatct atcataagta gaagccctgc tccatgaccc ctgcatttaa ataacgttt	5820
gttaaatgag tcaaattccc tcacatgag agctcacctg tgtgtaggcc catcacacac	5880
acaaacacac acacacacac acacacacac acacacacac acagggaaag tgcaggatcc	5940
tggacagcac caggcaggct tcacaggcag agcaaacagc gtgaatgacc catgcagtgc	6000
cctgggcccc atcagctcag agaccctgtg agggctgaga tggggctagg caggggagag	6060
acttagagag ggtggggcct ccagggaggg ggctgcaggg agctgggtac tgccctccag	6120
ggagggggct gcagggagct ggggtactgcc ctccagggag ggggctgcag ggagctgggt	6180
actgccctcc agggaggggg ctgcagggag ctgggtactg ccctccaggg agggggctgc	6240
agggagctgg gtactgccct ccagggaggc aggagcactg ttcccaacag agagcacatc	6300
ttcctgcagc agctgcacag acacaggagc ccccatgact gccctgggcc aggggtgtgga	6360
ttccaaattt cgtgccccat tgggtgggac ggaggtgac cgtgacatcc aaggggcatc	6420
tgtgattcca aacttaaact actgtgccta caaatagga aataacccta ctttttctac	6480
tatctcaaat tccctaagca caagctagca ccctttaaat caggaagtgc agtcactcct	6540
ggggtcctcc catgccccca gtctgacttg caggtgcaca ggggtggctga catctgtcct	6600
tgctcctcct cttggctcaa ctgccgcccc tctgggggt gactgatggt caggacaagg	6660
gacctagag ctggcccat gattgacagg aaggcaggac ttggcctcca ttctgaagac	6720
taggggtgtc aagagagctg ggcattccac agagctgcac agatgacgc ggacagaggg	6780

30/57

FIGURE 23E

tgacacaggg ctcagggctt cagacgggtc gggaggctca gctgagagtt caggacaga	6840
cctgaggagc ctcagtggga aaagaagcac tgaagtggga agttctggaa tgttctggac	6900
aagcctgagt gctctaagga aatgctccca ccccgatgta gcctgcagca ctggacggtc	6960
tgtgtacctc cccgctgccc atcctctcac agccccgcc tctagggaca caactcctgc	7020
cctaacatgc atctttcctg tctcattcca cacaaaagg cctctggggg cctgttctg	7080
cattgcaagg agtggaggtc acgttcccac agaccacca gcaacagggt cctatggagg	7140
tgcggtcagg aggatcacac gtcccccat gccaggga ctgactctgg ggggatgga	7200
ttggcctgga ggccactgg cccctctgtc cctgaggga atctgcaccc tggaggctgc	7260
cacatccctc ctgattcttt cagctgaggg cccttcttga aatcccaggg aggactcaac	7320
ccccactggg aaaggcccag tgtggacggg tccacagcag ccagctaag gcccttggac	7380
acagatcctg agtgagagaa cctttaggga cacaggtgca cggccatgtc cccagtgcc	7440
acacagagca ggggcatctg gaccctgagt gtgtagctcc cgcgactgaa cccagccctt	7500
cccaatgac gtgaccctg ggggtggctcc aggtctccag tccatgccac caaatctcc	7560
agattgaggg tcctcccttg agtccctgat gcctgtccag gagctgcccc ctgagcaaat	7620
ctagagtgca gagggtggg attgtggcag taaaagcagc cacatttgtc tcaggaagga	7680
aaggaggagc atgagctcca ggaaggcgga tggcgctctc tagtgggcgc ctctgttaa	7740
tgagcaaaaa ggggcccagga gagttgagag atcagggtc gccttggact aaggctcaga	7800
tggagaggac tgaggtgcaa agagggggct gaagtagggg agtggtcggg agagatggga	7860
ggagcaggta aggggaagcc ccaggaggc cgggggaggg tacagcagag ctctccactc	7920
ctcagcattg acatttgggg tggctcgtgct agtgggggtc tgtaagttgt aggggttca	7980
gcaccatctg gggactctac ccactazatg ccagcaggac tccctcccca agctctaaca	8040
accaacaatg tctccagact ttccaaatgt cccctggaga gcaaaattgc ttctggcaga	8100
atcactgate tacgtcagtc tctaaaagtg actcatcagc gaaatccttc acctctggg	8160
agaagaatca caagtgtgag aggggtagaa actgcagact tcaaatctt tccaaaagag	8220
ttttacttaa tcagcagttt gatgtcccag gagaagatac atttagagtg tttagagttg	8280
atgccacatg gctgcctgta cctcacagca ggagcagagt gggttttcca agggcctgta	8340
accacaactg gaatgacact cactgggtta cattacaaag tggaatgtgg ggaattctgt	8400
agactttggg aagggaatg tatgacgtga gccacagcc taaggcagtg gacagtccac	8460
tttgaggctc tcaccatcta ggagacatct cagccatgaa catagccaca tctgtcatta	8520

31/57

FIGURE 23F

gaaaacatgt- ttt attaaga ggaaaaatct aggc ta gaag tgctt at gc tcttttttct	8580
ctttatgttc aaattcatat acttttagat ctttcttaa agaagaatct atccccctaa	8640
gtaaatgtta t act gactg gatagtgtg gtgtctcact cccaaccct gtgtggtgac	8700
agtgcctgc tccccagcc ctgggccctc tctgattcct gagagcttg ggtgctcctt	8760
cattaggagg aagagaggaa ggggtgtttt aatattctca ccattcacc atccacctct	8820
tagacactgg gaagaatcag ttgccactc ttggatttga tctcgaatt aatgacctct	8880
atttctgtcc ctgtccatt tcaacaatgt gacaggccta agagggtgct tctccatgtg	8940
at ttt tgagg agaaggttct caagataagt tttctcacac ctcttgaat tacctccacc	9000
tgtgtcccca tcaccattac cagcagcatt tggaccctt ttctgttagt cagatgcttt	9060
ccacctcttg aggggtgtata ctgtatgctc tctacacagg aatatgcaga ggaaatagaa	9120
aaagggaaat cg catt acta ttcagagaga agaagacctt tatgtgaatg aatgagagtc	9180
taaaatccta agagagccca tataaaatta ttaccagtgc taaaactaca aaagttacac	9240
taacagtaaa ctagaataat aaaacatgca tcacagttgc tggtaagct aaatcagata	9300
tttttttctt agaaaaagca ttccatgtgt gttgcagtga tgacaggagt gcccttcagt	9360
caatatgctg cctgtaattt ttgttcctg gcagaatgta ttgtcttttc tccctttaa	9420
tcttaa at gc aaaactaaag gcagctcctg ggccccctcc ccaagtcag ctgcctgcaa	9480
ccagccccac gaagagcaga ggcctgagct tccctggtca aaatagggg ctagggagct	9540
taaccttgct cgataaagct gtgttcccag aatgtcgtc ctgttcccag gggaaccagc	9600
ctggaggggtg gtgagcctca ctggtggcct gatgcttacc ttgtgcctc acaccagtgg	9660
tactggaac cttgaacact tggctgtgc ccgatctgc agatgtcaag aacttctgga	9720
agtcaaatta ctgccactt ctccaggga gatacctgtg aacatccaaa accatgccac	9780
agaacctgc ctgggttcta caacacatat ggactgtgag caccaagtcc agccctgaat	9840
ctgtgaccac ctgccaagat gccctaact gggatccacc aatcactgca catggcaggc	9900
agcgaggctt ggaggtgctt cgccacaagg cagccccaat ttgctgggag tttcttgga	9960
cctggtagtg gtgaggagcc ttgggacct caggattact ccccttaagc atagtgggga	10020
cccttctgca tccccagcag gtgccccgt cttcagagcc tctctctctg aggtttaccc	10080
agacctgc accaatgaga ccatgctgaa gcctcagaga gagagatgga gctttgacca	10140
ggagccgctc ttccttgagg gccagggcag ggaaagcagg aggcagcacc aggagtggga	10200

32/57

FIGURE 23G

acaccagtgt ctaagcccct gatgagaaca ggggtggtctc tcccatatgc ccataccagg	10260
cctgtgaaca gaatcctcct tctgcagtga caatgtctga gaggacgaca tgtttcccag	10320
cctaacgtgc agccatgccc atctaccacac tgcctactgc aggacagcac caaccaggga	10380
gctgggaagc tgggagaaga catggaatac ccatggcttc tcaccttccct ccagtccagt	10440
gggcaccatt tatgcctagg acacccacct gccggcccca ggctcttaag agttaggtca	10500
cctaggtgcc tctgggaggc cgaggcagga gaattgcttg aaccggggag gcagaggttg	10560
cagtgaagcc agatcacacc actgcactcc agcctgggtg acagaatgag actctgtctc	10620
aaaaaaaaag agaaagatag catcagtggc taccaagggc taggggcagg ggaaggtgga	10680
gagttaatga ttaatagtat gaagtttcta tgtgagatga tgaaaatgtt ctggaaaaaa	10740
aaatatagtg gtgaggatgt agaatattgt gaataaatt aacggcattt aattgtacac	10800
ttaacatgat taatgtggca tattttatct tatgtatttg actacatcca agaaacactg	10860
ggagagggaa agcccaccat gtaaaataca cccaccctaa tcagatagtc ctcatgtac	10920
ccaggtacag gccctcatg acctgcacag gaataactaa ggatttaagg acatgaggct	10980
tcccagccaa ctgcaggtgc acaacataaa tgtatctgca aacagactga gagtaaagct	11040
gggggcacaa acctcagcac tgccaggaca cacacccttc tcgtggattc tgactttatc	11100
tgaccgggcc cactgtccag atcttggtgt gggattggga caaggagggt cataaagcct	11160
gtccccaggc cactctgtgt gagcacacga gacctcccc cccccacc gttaggtctc	11220
cacacataga tctgaccatt aggcattgtg aggaggactc tagcgcgggc tcagggatca	11280
caccagagaa tcagggtacag agaggaagac ggggctcgag gagctgatgg atgacacaga	11340
gcagggttcc tgcagtccac aggtccagct caccctgggt taggtgcccc atccccctga	11400
tccaggcatc cctgacacag ctccctcccg gagcctctc ccaggtgaca catcagggtc	11460
cctcactcaa gctgtccaga gagggcagca ccttgagacag cggccacccc acttcactct	11520
tcctccctca cagggtcag ggctcagggc tcaagtctca gaacaaatgg cagaggccag	11580
tgagcccaga gatggtgaca gggcaatgat ccaggggcag ctgcctgaaa cgggagcagg	11640
tgaagccaca gatgggagaa gatggttcag gaagaaaaat ccagggaatgg gcaggagagg	11700
agaggaggac acaggctctg tggggctgca gcccaggatg ggactaagtg tgaagacatc	11760
tcagcaggtg aggccaggtc ccatgaacag agaagcagct cccacctccc ctgatgcacg	11820
gacacacaga gtgtgtggtg ctgtgcccc agagtcgggc tctcctgttc tgggtcccag	11880
ggagtgaagaa gtgaggttga cttgtccctg ctctctctctg ctaccccaac attcaccttc	11940

33/57

FIGURE 23H

tccatcatgcc-cctctctctc	aaatatgatt	tygatctatg	tccccgccca	aatctcatgt	12000
caaattgtaa	acccaatgt	tggaggtggg	gccttgtag	aagtgattgg	12060
tggattttct	gctttgatgc	tgtttctgtg	atagagatct	cacatgatct	12120
aagtgtgtag	cacctctccc	ctctctctct	ctctctctta	ctcatgctct	12180
acgttcctgt	ttccccctca	ccgtccagaa	tgattgtaag	ttttctgagg	12240
agcagaagcc	actatgcttc	ctgtacaact	gcagaatgat	gagcgaatta	12300
ctttataaat	taccagctct	caggatatttc	tttatagcaa	tgcgaggaca	12360
atcttctact	cccagatccc	cgcacacgt	tagccccaga	catcactgcc	12420
tgacacagcg	agcctcctgc	cgacaaaagc	aaagtcacaa	aaggtgacaa	12480
ttggggacat	ctgattgtga	aagagggagg	acagtacact	tgtagccaca	12540
ctcaccgagc	tgaaacctgg	tagcactttg	gcataacatg	tgcatgaccc	12600
tctagagatc	agtgttgagt	aaaacagcct	ggctctggggc	cgctgctgtc	12660
tccgtgtccac	cagagggcgg	cagagttcct	cccaccctgg	agcctcccca	12720
acctccctca	gccgggcccc	cagcccagca	gggtccaccc	tcacccgggt	12780
cacgtccctc	tcgccctccg	agctcctcac	acggactctg	tcagctcctc	12840
atcgcccgcc	cacctgaggc	ttgtcggccg	cccacttgag	gcctgtcggc	12900
aggcagctcc	tgtcccttac	acccctcct	tccccgggt	cagctgaaag	12960
agggcagctc	cctgtgatct	ccaggacagc	tcagtctctc	acaggctccg	13020
tgctgtcacc	tcacagccct	gtcattacca	ttaaactcctc	agtcccatga	13080
gcgcctgtct	cccggttaca	ggaaaactct	gtgacagggg	ccacgtctgt	13140
gtggaatccc	aggggccagc	ccagtgcctg	acacggaaca	gatgtccat	13200
taaatgtgtg	ggagatctct	aaaaagaagc	atatcacctc	cgtgtggccc	13260
gagtctgttc	catgtggaca	caggggcact	ggcaccagca	tgggaggagg	13320
cccgcggctg	ccccaggaat	gaggcctcaa	ccccagagc	ttcagaaggg	13380
cctgcaggga	atagatcctc	cggcctgacc	ctgcagccta	atccagagtt	13440
tcacaccacg	tcgacctggg	tcagcatccc	tagggcagtt	ccagacaagg	13500
cctcttgccc	tccagggggt	gacattgcac	acagacatca	ctcaggaaac	13560
ggacaggaac	ctggctttgc	taagggaagtg	gaggtggagc	ctggtttcca	13620

34/57

FIGURE 23I

caacagaccc ttctgatctc tcccacatac ctgctctgtt cctttctcgg tcctatgagg	13680
accctgttct gccaggggtc cctgtgcaac tccagactcc ctctcggtag caccatgggg	13740
aaggtggggt gatcacagga cagtcagcct cgcagagaca gagaccaccc aggactgtca	13800
gggagaacat ggacaggccc tgagccgcag ctacagccaac agacacgcag agggaggggtc	13860
cccctggagc cttccccaag gacagcagag cccagagtca cccacctccc tccaccacag	13920
tcctctcttt ccaggacaca caagacacct cccctccac atgcaggatc tggggactcc	13980
tgagacctct gggcctgggt ctccatccct gggtcagtgg cggggttggg ggtactggag	14040
acagagggtc ggtccctccc cagccaccac ccagtgcgc tttttctagc ccccagagcc	14100
acctctgtca ccttctgtt gggcatcatc ccacctccc agagccctgg agagcatggg	14160
gagacccggg accctgctgg gtttctctgt cacaaaggaa aataatcccc ctgggtgtgac	14220
agacccaagg acagaacaca gcagaggtca gcactgggga agacagggtg tcctcccagg	14280
ggatgggggt ccatccacct tgccgaaaag atttgtctga ggaactgaa atagaaggga	14340
aaaagagga gggacaaaag aggcagaaat gagaggggag gggacagagc acacctgaat	14400
aaagaccaca cccatgaccc acgtgatgct gagaagtact cctgccctag gaagagactc	14460
aggccagagg gaggaaggac agcagaccag acagtcacag cagccttgac aaaacgttcc	14520
tggaactcaa gctcttctcc acagaggagg acagagcaga cagcagagac catggagtct	14580
ccctcggccc ctccccacag atgggtgcatc ccctggcaga ggcctctgct cacagggtgaa	14640
gggaggacaa cctgggagag ggtgggagga gggagctggg gtctcctggg taggacaggg	14700
ctgtgagacg gacagagggc tcctgttggg gcctgaatag ggaagaggac atcagagagg	14760
gacaggagtc acaccagaaa aatcaaatg aactggaatt ggaaggggc aggaaacct	14820
caagagttct attttcttag ttaattgtca ctggccacta cgttttttaa aatcataata	14880
actgcatcag atgacacttt aaataaaaac ataaccaggg catgaaacac tgtcctcatc	14940
cgcctaccgc ggacattgga aaataagccc caggctgtgg agggccctgg gaacctcat	15000
gaactcatcc acaggaatct gcagcctgtc ccaggcactg gggtgcaacc aagatc	15056

35/57

FIGURE 24A

gaattcagaa ataggggaag gttgaggaag gacactgaac tcaaagggga tacagtgtatt 60
ggtttatttg tcttctcttc acaacattgg tgctggagga attcccaccc tgagggtatg 120
aagatgtctg aacacccaac acatagcact ggagatatga gctcgacaag agtttctcag 180
ccacagagat tcacagccta gggcaggagg aactgtacg ccaggcagaa tgacatggga 240
attgcgctca cgattggctt gaagaagcaa ggactgtggg aggtgggctt tgtagtaaca 300
agagggcagg gtgaactctg attcccatgg gggaatgtga tggtcctglt acaaattttt 360
caagctggca gggaataaaa cccattacgg tgaggacctg tggagggcgg ctgccccaac 420
tgataaagga aatagccagg tgggggcctt tcccattgta ggggggadat atctggcaat 480
agaagccttt gagacccttt aggggtacaag tactgaggca gcaaataaaa tgaaatctta 540
tttttcaact ttatactgca tgggtgtgaa gatataattg tttctgtaca gggggtgagg 600
gaaaggaggg gaggaggaag gttcctgcag gtctggtttg gtcttctgat ccaggggggtc 660
ttggaactat ttaazttaaa ttaaatataa acaagcgact gttttaaatt aaattaaatt 720
aaattaaatt ttactttatt ttatcttaag ttctgggcta catgtgcagg acgtgcagct 780
ttgttacata ggtaaacgtg tgccatggtg gtttgctgta cctatcaacc catcacctag 840
gtattaagcc cagcatgcat tagctgtttt tctgacgct ctccctctcc ctgactccca 900
caacaggccc cagtgtgtgt tgttcccctc cctgtgtcca tgtgttctca ttgttcagct 960
cccacttata agtgagaaca tgtggtgttt ggttttctgt ttctgtgtta gtttgctgag 1020
gataatggct tccacctcca tccatgttcc tgcaaaggac gtgatcttat tcttttttat 1080
ggttgcatag aaattgtttt tacaaatcca attgatattg tatttaatta caagttaatc 1140
taattagcat actagaagag attacagaag atattaggta cattgaatga ggaaatatat 1200
aaaataggac gaagggtgaaa tattaggtag gaaaagtata atagttgaaa gaagtaaaaa 1260
aaaatatgca tgagtagcag aatgtaaaag aggtgaagaa cgtaatagtg actttttaga 1320
ccagattgaa ggacagagac agaaaaattt taaggaattg ctaaaccatg tgagtgttag 1380
aagtacagtc aataacatta aagcctcagg aggagaaaag aataggaaag gaggaaatat 1440
gtgaataaat agtagagaca tgtttgatgg attttaaaat atttgaaaga cctcacatca 1500
aaggattcat accgtgccat tgaagaggaa gatggaaaag ccaagaagcc agatgaaagt 1560
tagaaatatt attggcaaag cttaaatgtt aaaagtccta gagagaaagg atggcagaaa 1620
tattggcggg aaagaatgca gaacctagaa tataaattca tccaacagt ttggtagtgt 1680

36/57

FIGURE 24B

gcagctgtag ccttttctag ataatacact attgtcatac atcgcttaag cgagtgtaaa 1740
 atgggtctcct cactttattt atttatatat ttatttagtt ttgagatgga gcctcgctct 1800
 gtctcctagg ctggagtga atagtgcgat accactcact gcaacctctg cctcctctgt 1860
 tcaagtgatt ttcttacctc agcctcccgga gtagctggga ttacagggtgc gtgccaccac 1920
 acccggtctaa tttttgtatt tttttagtag acgggggttt gccatgttgg ccaggctggt 1980
 cttgaactcc tgacatcagg tgatccacct gccttggcct cctaaagtgc tgggattaca 2040
 ggcatgagcc accgtgcccc accactttat ttatttttta tttttatatt taaatttcag 2100
 cttctatttg aaatacaggg ggcacatata taggattggt acatgggtat attgaactca 2160
 ggtagtgatc atactacca acaggtaggt tttcaacca ctccccctct tttctcccc 2220
 attctagtag tgtgcagtgt ctattgttct catgtttatg tctatgtgtg ctccagggtt 2280
 agtccccacc tgtaagtga aacgtgtggt atttgatttt ctgtccctgt gtaattcac 2340
 ttaggattat ggcttccagc tccattcata ttgctgtaaa ggatatgatt catttttcat 2400
 ggccatgcag tattccatat tgcgtataga tcacattttc tttctttttt tttttgaga 2460
 cggagtcttg ctttgctgcc taggctggag tgcagtagca cgatctcggc tcaactgcaag 2520
 cttcacctcc ggggttcacg tcattcttct gtctcagctt cccaagtagc tgggactaca 2580
 ggcgcccgcc accacgtccg gctaattttt ttgtgtgttt ttagtagaga tgggggtttc 2640
 actgtgttag ccaggatggt cttgatctcc tgacctgtg gtccacctgc ctcggtctcc 2700
 caaagtgtcg ggattacagg ggtgagccac tgcgcccggc ccatatatac cacattttct 2760
 ttaaccaatc caccattgat gggcaactag gtagattcca tggattccac agttttgcta 2820
 ttgtgtgcag tgtggcagta gacatatgaa tgaatgtgtc tttttggtat aatgatttgc 2880
 attccttttg gtatacagtc attaatagga gtgctgggtt gaacggtggc tctgtttaaa 2940
 attcctttgag aattttccaa actgtttgcc atagagagca aactaattta catttccacg 3000
 aacagtatat aagcattccc tttctccac agctttgtca tcatgggttt ttttttctt 3060
 tattttaaaa aagaatatgt tgttgttttc ccagggtaca tgtgcaggat gtgcagggtt 3120
 gttacatagg tagtaaactg gagccatggt ggtttgtgc acctgtcaac ccattacctg 3180
 ggtatgaagc cctgcctgca ttagctcttt tccctaagtc tctactact gccccacct 3240
 caccctgaca gggcaaacag acaacctaca gaatgggagg aaatttttgc aatctattca 3300
 tctgacaaag gtcaagaata tccagaatct acaaggaaact taagcaaatt tttactttt 3360

37/57

FIGURE 24C

aataatagcc actctgactg gcgtgaaatg ctatctcatt gtggttttca tttgaatttc 3420
tctgatgatc agtgacgatg agcatttttt catatttggt ggctgcttgt acgtcttttg 3480
agaagtgtct cttcatgcct tttggccact ttaatgggat tattttttgc tttttagttt 3540
aagttcctta tagattctgg atattagact tcttattgga tgcatagttt gtgaatactc 3600
tcttccattc tgtaggttgt ctgtttactc tattgatggc ttcttttgct gtgccgaagc 3660
atcttagttt aattagaaac cacctgccaa tttttgtttt tgttgcaatt gcttttgggg 3720
acttagtcat aaactctttg ccaaggctcg ggtcaagaag agtatttcct aggttttctt 3780
ctagaatttt gaaagtctga atgtaaacad ttgcattttt aatgcattct gagttagttt 3840
ttgtatatgt gaaaggctca ctctcatttt cttccctctt ttctttcttt cttcttttc 3900
tttctttctt tctttctttc tttctttctt tctttctttc tttctttttg tcttctttc 3960
tttctttctt tctctttctt tctctctttc tttttttttt ttgatggagt attgctctgt 4020
tgcccaggct gcagtgcagc ggacgatct cggctcactg caacctctgc ctctgggtt 4080
caactgattc tctgcatca gccttccaag tagctgggat tataggcgcc cgccaccacg 4140
cccgaactaa ttttgtattt ttagtagaga cggggttggt ccatgttggc caggctgggt 4200
tgaaactcct gacctcaaac gatctgcctg ccttggcctc ccaaagtgtt gggattacag 4260
gtgtgagcca ctgtgccag ccaagaatgt cattttctaa gaggtccaag aacctcaaga 4320
tattttggga ccttgagaag agaggaattc atacaggtat tacaagcaca gcctaattgc 4380
aaatctttgg catggcttgg cttcaagact ttaggtcttt aaaagtcgaa tccaaaaatt 4440
tttataaaag ctccagctaa gctaccttaa aeggggcctg tatggctgat cactcttctt 4500
gctatacttt acacaaataa acaggccaaa tataatgagg ccaaaattta ttttgcaaat 4560
aaattggtcc tgctatgatt tactcttggg aagaacaggg aaaatagaga aaaatttaga 4620
ttgcatctga cctttttttc tgaattttta tatgtgccta caatttgagc taaatcctga 4680
attattttct gggtgcaaaa actctctaaa gaagaacttg gttttcattg tcttcgtgac 4740
acatttatct ggctctttac tagaacagct ttcttgttt tgggtgttcta gcttgtgtgc 4800
cttacagttc tactcttcaa attattgtta tgtgtatctc atagttttcc ttcttttgag 4860
aaaactgaag ccatggtatt ctgaggacta gagatgactc aacagagctg gtgaatctcc 4920
tcatatgcaa tccactgggc tcgatctgct tcaaatgct gatgcactgc tgctaaagct 4980
atacatttta aaccctcact aaaggatcag ggaccatcat ggaagaggag gaaacatgaa 5040
attgtaagag ccagattcgg ggggtagagt gtggagggtca gagcaactcc accttgaata 5100

38/57

FIGURE 24D

agaaggtaaa gcaacctatc ctgaaagcta aectgccatg gtggcttctg attaacctct 5160
 gttctaggaa gactgacagt ttgggtctgt gtcattgccc aaatctcatg ttaaattgta 5220
 atccccagtg ttccggaggtg ggacttggtg gtaggtgatt cggtcatggg agtagatttt 5280
 cttctttgtg gtgttacagt gatagtgagt gagttctcgt gagatctggt catttaaaag 5340
 tgtgtggccc ctccccctcc tctcttggtc ctctactgc catgtaagat acctgctcct 5400
 gctttgcctt ctaccataag taaaagcccc ctgaggcctc ccagaagca gatgccacca 5460
 tgcttctgt acagcctgca gaaccatcag ccaattaaac ctcttttctg tataaattac 5520
 cagtcttgag tatctcttta cagcagtgtg agaacggact aatacaaggg tctccaaaat 5580
 tccaagttha tgtattcttt cttgccaaat agcaggtatt taccataaat cctgtcctta 5640
 ggtcaaacaa ccttgatggc atcgtacttc aattgtctta cacattcctt ctgaatgact 5700
 cctcccctat ggcatataag ccctgggtct tgggggataa tggcagaggg gtccaccatc 5760
 ttgtctggct gccacctgag acacggacat ggttctgtt ggtaagtctc tattaaatgt 5820
 ttctttctaa gaaactggat ttgtcagctt gtttctttgg cctctcagct tctcagact 5880
 ttggggtagg ttgcacaacc ctgcccacca cgaaapaaat gtttaatatg ataaatatgg 5940
 atagatataa tccacataaa taaaagctct tggaggggccc tcaataattg ttaagagtgt 6000
 aaatgtgtcc aaagatggaa aatgtttgag aactactgc ccagagattt tcttgagttc 6060
 tagagtgtgg gaatatagaa cctggagctt ggcttcttca gcctagaatc aggagtatgg 6120
 ggctgaagtc tgaagcttgg cttcagcagt tgggggttgg cttccggagc acatatttga 6180
 catgttgcca ctgtgatttg gggtttggtt ttgtctctga atcctaattg ctgtccttga 6240
 ggcatctaga atctgaaatc tgtggtcaga attctattat cttgagtagg acatctccag 6300
 tcttggttct gccttctagg gctggagtct gtagtcagtg acccggtctg gcatttcaac 6360
 ttcatataca gtgggctatc ttttgggtcca tgtttcaacc aaacaaccga ataaaccatt 6420
 agaacctttc ccacttccc tagctgcaat gttaaacctt ggatttctgt ttaatagggt 6480
 catatgaata atttcagcct gatccaactt tacattcctt ctaccgttat tctacacca 6540
 ccttaaaaat gcattcccaa tatattccct ggattctacc tatatatggt aatcctgggt 6600
 ttgccagttt ctagtgcatt aacatacctg atttacattc ttttacttta aagtggaaat 6660
 aagagtcctt ctgcagagtt caggagttct caagatggcc cttacttctg acatcaattg 6720
 agatttcaag ggagtcgcca agatcatcct caggttcagt gattgctggt agccctcata 6780

FIGURE 24E

taactcaatg aaagctgtta tgctcatggc tatggtttat tacagcaaaa gaatagagat 6840
 gaaaatctag caaggggaaga gttgcatggg gcaaagacaa ggagagctcc aagtgcagag 6900
 attcctgttg ttttctccca gtggtgtcat ggaaagcagt atcttctcca tacaatgatg 6960
 tgtgataata ttcagtgtat tgccaatcag ggaactcaac tgagccttga ttatattgga 7020
 gcttggttgc acagacatgt cgaccacctt catggctgaa ctttagtact tagcccctcc 7080
 agacgtctac agctgatagg ctgtaaccca agattgtcac cataaatcac attggttagac 7140
 tatccagtgt gggccaagct cccgtgtaaa cacaggcact ctaaacaggc aggatatttc 7200
 aaaagcttag agatgacctc ccaggagctg aatgcaaaga cctggcctct ttgggcaagg 7260
 agaatecttt accgcacact ctcttcaca gggttattgt gaggatcaaa tgtggtcatg 7320
 tgtgtgagac accagcacat gtctggctgt ggagagtgaac ttctatgtgt gctaacattg 7380
 ctgagtgtca agaaagtatt aggcattggc ttgagcactc acagatgtct atctaactct 7440
 cacaacatgg ctacaggggt ggcactacta gcctcatttg acagaggaaa ggactgtgga 7500
 taagaagggg gtgaccaata ggtcagagtc attctggatg caaggggctc cagaggacca 7560
 tgattagaca ttgtctgcag agaaattatg gctggatgtc tctgccccgg aaagggggat 7620
 gcactttcct tgacccccca tctcagatct tgactttgag gttatctcag acttcctcta 7680
 tgataccagg agcccatcat aatctctctg tgctctctcc ccttcctcag tcttactgcc 7740
 cactcttccc agctccatct ccagctggcc aggtgtagcc acagtaccta actctttgca 7800
 gagaactata aatgtgtatc ctacagggga gaaaaaaaaa aagaactctg aaagagctga 7860
 cattttaccg acttgcaaac acataagcta acctgccagt tttgtgctgg tagaactcat 7920
 gagactcctg ggtcagaggc aaaagatttt attaccaca gctaaggagg cagcatgaac 7980
 tttgtgttca catttgttca ctttgcccc caattcatat gggatgatca gagcagttca 8040
 ggtggatgga cacagggggt tgtggcaaaag gtgagcaacc taggcttaga aatcctcaat 8100
 cttataagaa ggtactagca aactgtcca gtctttgtat ctgacggaga tattatcttt 8160
 ataattgggt tgaaagcaga cctactctgg aggaacatat tgtattttatt gtctgaaca 8220
 gtaaacaaat ctgctgtaaa atagacgtta actttattat ctaaggcagt aagcaaacct 8280
 agatctgaag gcgataccat cttgcaaggc tatctgctgt acaaatatgc ttgaaaagat 8340
 ggtccagaaa agaaaacggt attattgcct ttgctcagaa gacacacaga aacataagag 8400
 aaccatggaa aattgtctcc caacactgtt caccagagc cttccactct tgtctgcagg 8460
 acagtcttaa catcccatca ttagtgtgtc taccacatct ggcttcaccg tgcctaacca 8520

40/57

FIGURE 24F

```

agatttctag gtccagttcc ccacatggt tggcagtgcc ccactgccaa cccagaata 8580
agggagtgc cagaattccg aggggacatg ggtggggatc agaacttctg ggcttgagtg 8640
cagagggggc ccatactcct tggttccgaa ggaggagag gctggagggtg aatgtccttg 8700
gaggggagga atgtgggttc tgaactctta aatccccaag ggaggagact ggtaagggtcc 8760
cagcttccga ggtactgacg tgggaatggc ctgagagggtc taagaatccc gtatcctcgg 8820
gaaggagggg ctgaaattgt gaggggttga gttgcagggg tttgttagct tgagactcct 8880
tggtgggtcc ctgggaagca aggactggaa ccattgggtc cagggttttg tgtgaaggta 8940
atgggatctc ctgattctca aagggtcaga ggactgagag ttgccatgc tttgatcttt 9000
ccatctactc cttactccac ttgagggtaa tcacctactc ttctagttcc acaagagtgc 9060
gcctgcgcga gtataatctg cacatgtgcc atgtcccag gctggggca tcatccactc 9120
atcattcagc atctgcgcta tgcgggcgag gccggcgcca tgacgtcatg tagctgcgac 9180
tatccctgca gcgcgcctct cccgtcacgt cccaaccatg gagctgtgga cgtgcgtccc 9240
ctggtggatg tggcctgcgt ggtgccaggc cggggcctgg tctccgataa agatcctaga 9300
accacaggaa accaggactg aaagggtgcta gagaatggcc atatgtcgct gtccatgaaa 9360
tctcaaggac ttctgggttg agggcacagg agcctgaact tacggggttg cccagtgcca 9420
ctgtcctccc aagtgagtct ccagatacg aggcactgtg ccagcatcag cttcatctgt 9480
accacatctt gtaacaggga ctaccaggga ccctgatgaa caccatggtg tgtgcaggaa 9540
gagggggtga aggcattggc tcctgtgtgg tcagagccca gagggggcca tgacgggtgg 9600
ggaggaggct gtggactggc tcgagaagtg ggatgtggtt gtgtttgatt tcctttggcc 9660
agataaagtg ctggatatag cattgaaaac ggagtatgaa gaccagttag aatggagggt 9720
caggttgag ttgagttaca gatggggtaa aattctgctt ccgatgagtt tggggattgg 9780
caatctaaag gtggtttggg atggcatggc tttgggatgg aataggttt gtttttatgt 9840
tggctgggaa ggggtgtggg attgaattgg ggatgaagta ggttagttt tggagataga 9900
atacatggag ctggctattg catgcgagga tgtgcattag ttgggtttga tctttaaata 9960
aaggaggcta ttagggttgt cttgaattag attaagttgt gtggggttga tgggttgggc 10020
ttgtgggtga tgtgggttga ttgggctgtg ttaaattggt ttgggtcagg ttttggttga 10080
ggttatcatg gggatgagga tatgcttggg acatggattc aggtggttct cattcaagct 10140
gaggcaaatt tcctttcaga cggtcattcc agggaacgag tgggtgtgtg ggggaaatca 10200

```

41/57

FIGURE 24G

ggccactggc tgtgaatata cctctatcct ggtcttgaat tgtgattata tatgtccatt 10260
ctgtctcctt cactgtactt ggaattgata tggtcattca gctggaaatg ggggaagatt 10320
ttgtcaaatt cttgagacac agctgggtct ggatcagcgt aagccttctt tctggtttta 10380
ttgaacagat gaaatcacat tttttttttt aaaatcacag aaatcttata gagttaacag 10440
tggactctta taataagagt taacaccagg actcttattc ttgattcttt tctgagacac 10500
caaatgaga tttctcaatg ccaccctaatt totttttttt tttttttttt tttttgagac 10560
acagtctggg tcttttgctc tgtcactcag gctggagcgc agtgggtgta tcatagctca 10620
ctgaaccctt gacctcctgg acttaaggga tctcctgct tcagcctcct gagtagatgg 10680
ggctacaggt gcttgccacc acacctggct aattaaattt tttttttttt tttgtagaga 10740
aagggtctca ctttggtgcc ctggctgata ttgaacttct gacttcaagt gattcttcag 10800
ccttggactc ccaaagcact gggattgctg gcatgagcca ctcaccgtgc ctggcttgca 10860
gcttaattct ggagtgtata aacctggctc ctgatagcta gacatttcag tgagaaggag 10920
gcattggatt ttgcatgagg acaattctga cctaggaggg caggtaaa ggaatccccg 10980
ctgtacctgt acgttggtaca ggcatggaga atgaggagtg aggaggccgt accggaaccc 11040
catattgttt agtggacatt ggattttgaa ataataggga acttgggtctg ggagagtcat 11100
atttctggat tggacaatat gtggtatcac aaggttttat gatgaggag aaatgtatgt 11160
ggggaaccat tttctgagtg tggaaagtga agaatacagag agtagctgaa tgccaacgct 11220
tctatttcag gaacatggta agttggaggt ccagctctcg ggctcagacg ggtataggga 11280
ccagggaagtc tcacaatccg atcattctga tatttcaggg catattaggt ttggggtgca 11340
aagggaagta ttgggactta ggcacatgag actttgtatt gaaaatcaat gattggggct 11400
ggccgtgggtg ctcacgctg taatctcatc actttgggag accgaagtgg gaggatggct 11460
tgatctcaag agttggacac cagcctaggc aacatggcca gacctctct ctacaaaaaa 11520
attaaaaatt agctggatgt ggtggtgcat gcttggtggtc tcagctatcc tggaggctga 11580
gacaggagaa tcggttgagt ctgggagttc aaggctacag ggagctgcga tcacgccgct 11640
gcactccagc ctgggaaaca gagtgaact gtctcagaat ttttttaaaa aagaatcagt 11700
gatcatccca accctgttg ctgttcaccc tgagcctgcc ttctctggct ttgttcccta 11760
gatcacatct ccatgatcca taggcctgac ccaatctgac ctcacaccgt gggaatgcct 11820
ccagactgat ctagtatgtg tggaaacagca agtgctggct ctccctcccc ttccacagct 11880
ctgggtgtgg gagggggttg tccagcctcc agcagcatgg ggagggcctt ggtcagcatc 11940

42/57

FIGURE 24H

taggtgcaa cagggcaagg gcggggctct ggagaatgaa ggctttatag ggctcctcag 12000
ggaggccccc cagccccaaa ctgcaccacc tggccgtgga caccggc 12047

43/57

FIGURE 25

cgagcggccc ctcagcttcg gcgcccagcc ccgcaaggct cccggtgacc actagagggc 60
gggaggagct cctggccagt ggtggagagt ggcaaggaag gaccctaggg ttcacgag 120
cccaggttta ctcccttaag tggaaatttc ttccccact cctccttggc tttctccaag 180
gaggggaaccc aggctgctgg aaagtccggc tggggcgggg actgtgggtt caggggagaa 240
cggggtgtgg aacgggacag ggagcggtta gaagggtggg gctattccgg gaagtgggtg 300
ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctcgg 360
ccgctctgct tcagtggacc cggggagggc ggggaagtgg agtgggagac ctagggtgg 420
gttcccgac cttgctgtac aggacctga cctagctggc ttgttcccc atccccacgt 480
tagttgttgc cctgaggcta aaactagagc ccaggggccc caagttccag actgccctc 540
ccccctcccc cggagccagg gagtggttgg tgaaaggggg aggccagctg gagaacaaac 600
gggtagtcag ggggttgagc gattagagcc cttgtaccct acccaggaat gggtggggag 660
gaggaggaag aggtaggagg taggggaggg ggcggggttt tgtcacctgt cacctgctcg 720
ctgtgcctag ggcgggaggc cggggagtgg ggggaccggt ataaagcggg aggcgcctgt 780
gcccgtcca cctctcaagc agccagcgcc tgctgaatc tgttctgccc cctccccacc 840
catttcacca ccaccatg 858

FIGURE 26

aagcttccac aagtgcattt agcctctcca gtattgctga tgaatccaca gttcaggttc 60
aatggcggtc aaaacttgat caaaaatgac cagactttat attcttacac caacatctat 120
ctgattggag gaatggataa tagtcatcat gtttaaacad ctaccattcc agttaagaaa 180
atatgatagc atcttgttct tagtcttttt cttaataggg acataaagcc cacaaataaa 240
aatatgcctg aagaatggga caggcattgg gcattgtcca tgcctagtaa agtactccaa 300
gaacctatct gtatactaga tgacacaatg tcaatgtctg tgtacaactg ccaactggga 360
tgcaagacac tgcccatgcc aatcatcctg aaaagcagct ataaaaagca ggaagctact 420
ctgcaccttg tcagtgaggt ccagatacct acag 454

45/57

FIGURE 27

GCATTGCTGT GAACTCTGTA CTTAGGACTA AACTTTGAGC AATAACACAC ATAGATTGAG
60

GATTGTTTGC TGTTAGCATA CAAACTCTGG TTCAAAGCTC CTCTTTATTG CTTGTCTTGG
120

AAAATTTGCT GTTCTTCATG GTTCTCTTT TCACTGCTAT CTATTTTTCT CAACCACTCA
180

CATGGCTACA ATAACTGTCT GCAAGCTTAT GATTCCCAA TATCTATCTC TAGCCTCAAT
240

CTTGTTCAG AAGATAAAAA GTAGTATTCA AATGCACATC AACGTCTCCA CTTGGAGGGC
300

TAAAGACGT TTCAACATAC AAACCGGGA GTTTGCCTG GAATGTTTCC TAAATGTGT
360

CCTGTAGCAC ATAGGGTCCT CTTGTTCTT AAAATCTAAT TACTTTTAGC CCAGTGCTCA
420

TCCACCTAT GGGGAGATGA GAGTGAAAAG GGAGCCGAT TAAATAATTAC ACTAAGTCAA
480

TAGGCATAGA GCCAGGACTG TTTGGGTAAA CTGGTCACTT TATCTTAAAC TAAATATATC
540

CAAACTGAA CATGTACTTA GTTACTAAGT CTTTGACTTT ATCTCATTCA TACCACTCAG
600

CTTTATCCAG GCCACTTATG AGCTCTGTGT CCTTGACAT AAATACAAA TAACCGCTAT
660

GCTGTTAATT ATTGGCAAAT GTCCCATTTT CAACCTAAGG AAATACCATA AAGTAACAGA
720

TATACCAACA AAAGGTTACT AGTTAACAGG CATTGCCTGA AAAGAGTATA AAAGAATTTC
780

AGCATGATTT TCCATATTGT GCTTCCACCA CTGCCAATAA CA
822

46/57

FIGURE 28A

GAATTCTTAG AAATATGGGG GTAGGGGTGG TGGTGGTAAT TCTGTTTCA CCCCATAGGT
60

GAGATAAGCA TTGGGTAA TGTGCTTTCA CACACACATC ACATTTCATA AGAATTAAGG
120

AACAGACTAT GGGCTGGAGG ACTTTGAGGA TGTCTGTCTC ATAACACTTG GGTGTATCT
180

GTTCTATGGG GCTTGTTTAA AGCTTGGCAA CTTGCAACAG GGTTCACTGA CTTTCTCCCC
240

AAGCCCAAGG TACTGTCCTC TTTTCATATC TGTTTTGGGG CCTCTGGGGC TTGAATATCT
300

GAGAAAATAT AAACATTTCATAAATGTTCT GTGGTGAGAT GAGTATGAGA GATGTGTCA
360

TCATTTGTAT CAATGAATGA ATGAGGACAA TTAGTGTATA AATCCTTAGT ACAACAATCT
420

GAGGGTAGGG GTGGTACTAT TCAATTTCTA TTTATAAAGA TACTTATTC TATTATTTA
480

TGCTTGTGAC AAATGTTTTG TTCGGGACCA CAGGAATCAC AAAGATGAGT CTTGAATTT
540

AAGAAGTTAA TGGTCCAGGA ATAATTACAT AGCTTACAAA TGACTATGAT ATACCATCAA
600

ACAAGAGGTT CCATGAGAAA ATAATCTGAA AGGTTTAATA AGTTGTCAA GGTGAGAGGG
660

CTCTTCTCTA GCTAGAGACT AATCAGAAAT ACATTCAGGG ATAATTATTT GAATAGACCT
720

TAAGGGTTGG GTACATTTTG TTCAAGCATT GATGGAGAAG GAGAGTGAAT ATTTGAAAAC
780

ATTTTCAACT AACCAACCAC CCAATCCAAC AAACAAAAA TGAAAAGAAT CTCAGAAACA
840

GTGAGATAAG AGAAGGAATT TTCTCACAAC CCACACGTAT AGCTCAACTG CTCTGAAGAA
900

GTATATATCT AATATTTAAC ACTAACATCA TGCTAATAAT GATAATAATT ACTGTCATTT
960

TTTAATGTCT ATAAGTACCA GGCATTAGA AGATATTATT CCATTTATAT ATCAAAATAA
1020

47/57

FIGURE 28B

ACTTGAGGGG ATAGATCATT TTCATGATAT ATGAGAAAAA TTAALAACAG ATTGAATTAT
1080

TTGCCGTGCA TACAGCTAAT AATTGACCAT AAGACAATTA GATTTAAATT AGTTTTGAT
1140

CTTTCTAATA CCAAAGTTCA GTTTACTGTT CCATGTTGCT TCTGAGTGGC TTCACAGACT
1200

TATGAAAAAG TAAACGGAAT CAGAATTACA TCAATGCAAA AGCATTGCTG TGAACTCTGT
1260

ACTTAGGACT AAACCTTGAG CAATAACACA CATAGATTEA GGATTGTTTG CTGTTAGCAT
1320

ACAACTCTG GTTCAAAGCT CCTCTTTATT GCTTGTCTTG GAAAATTTGC TGTCTTCAT
1380

GGTTTCTCTT TTCACTGCTA TCTATTTTTC TCAACCACTC ACATGGCTAC AATAACTCTC
1440

TGCAAGCTTA TGATTCCCAA ATATCTATCT CTAGCCTCA TCTTGTTCCA GAAGATAAAA
1500

AGTAGTATTC AAATGCACAT CAACGTCTCC ACTTGGAGGG CTTAAGACG TTCAACATA
1560

CAAACCGGGG AGTTTTGCCT GGAATGTTTC CTAAATGTC TCCTGTAGCA CATAGGGTCC
1620

TCTTGTTCTT TAAAATCTAA TTACTTTTAG CCCAGTGCTC ATCCACCTA TGGGGAGATG
1680

AGAGTGAAAA GGGAGCCTGA TTAATAATTA CACTAAGTCA ATAGECATAG AGCCAGGACT
1740

GTCTGGGTAA ACTGGTCACT TTATCTTAAA CTAAATATAT CCAAACTGA ACATGTACTT
1800

AGTTACTAAG TCTTGACTT TATCTCATT ATACCACTCA GCTTTATCCA GGCCACTTAT
1860

TTGACAGTAT TATTGCGAAA ACTTCCTAAC TGGTCTCCTT ATCATAGTCT TATCCCCTTT
1920

TGAAACAAAA GAGACAGTTT CAAAATACAA ATATGATTTT TATTAGCTCC CTTTGTGTGT
1980

CTATAATAGT CCCAGAAGGA GTTATAAACT CCATTTAAA AGTCITTGAG ATGTGGCCCT
2040

TGCCAACTTT GCCAGGAATT CCAATATCT AGTATTTTCT ACTATTAAAC TTTGTGCCTC
2100

TTCAAACTG CATTTTCTCT CATTCCCTAA GTGTGCATG TTTTCCCTTA CCGTTGGTT
2160

FIGURE 28C

TTTCCACCAC CTTTACATT TTCCTGGAAC ACTATACCCT CCCTCTTCAT TTGGCCCACC
2220

TCTAATTTTC TTTCAGATCT CCATGAAGAT GTTACTTCCT CCAAGGAAGCC TTATCTGACC
2280

CCTCCAAAGA TGTCATGAGT TCCTCTTTTC ATTCTACTAA TCAAGCATC CATCACACCA
2340

TGTTGTGATT ACTGATACTA TTGTCTGTTT CTCTGATTAG GCAGTAAGCT CAACAAGAGC
2400

TACATGGTGC CTGTCTCTTG TTGCTGATTA TTCCCA?CCA AAACAGTGC CTGGAATGCA
2460

GACTTAACAT TTTATTGAAT GAATAAATAA AACCCCATCT ATCGAGTGCT ACTTTGTGCA
2520

AGACCCGGTT CTGAGGCATT TATATTTATT GATTTA?TTA ATTCTCATTT AACCATGAAG
2580

GAGGTACTAT CACTATCCTT ATTTTATAGT TGATAAAGAT AAAGCCCAGA GAAATGAATT
2640

AACTCACCCA AAGTCATGTA GCTAAGTGAC AGGGCA?AAA TTCAAACCAG TTCCCCAACT
2700

TTACGTGATT AATACTGTGC TATACTGCCT CTCTGATCAT ATGSCATGGA ATGCAGACAT
2760

CTGCTCCGTA AGGCAGAATA TGGAAGGAGA TTGGAGGATG ACACAAAACC AGCATAATAT
2820

CAGAGGAAAA GTCCAAACAG GACCTGAACT GATAGAAAAG TTGTTACTCC TGGTGTAGTC
2880

GCATCGACAT CTTGATGAAC TGGTGGCTGA CACAACATAC ATTGGCTTGA TGTGTACATA
2940

TTATTTGTAG TTGTGTGTGT ATTTTATAT ATATATTTGT AATATTGAAA TAGTCATAAT
3000

TTACTAAAGG CCTACCATTG GCCAGGCATT TTTACATTTG TCCCCTCTAA TCTTTTGATG
3060

AGATGATCAG ATTGGATTAC TTGGCCTTGA AGATGATATA TCTACATCTA TATCTATATC
3120

TATATCTATA TCTATATCTA TATCTATATC TATATCTATA TATGTATATC AGAAAAGCTG
3180

AAATATGTTT TGTAAGTTA TAAAGATTTT AGACTTTATA GAATCTGGGA TTTGCCAAAT
3240

GTAACCCCTT TCTCTACATT AAACCCATGT TGAACAAAT ACATTTATTA TTCATTCATC
3300

49/57

FIGURE 28D

AAATGTTGCT GAGTCCTGGC TATGAACCAG ACACTGTGAA AGCCTTTGGG ATATTTTGCC
3360

CATGCTTGGG CAAGCTTATA TAGTTTGCTT CATAAACTC TATTTAGTT CTTCATACT
3420

AATACTTCAT GACTATTGCT TTTCAGGTAT TCCTTCATAA CAAATACTTT GGCTTTCATA
3480

TATTTGAGTA AAGTCCCCCT TGAGGAAGAG TAGAAGAACT GCACTTTGTA AATACTATCC
3540

TGGAATCCAA ACGGATAGAC AAGGATGGTG CTACCTCTTT CTGGAGAGTA CGTGAGCAAG
3600

GCCTGTTTTG TTAACATGTT CCTTAGGAGA CAAAACCTAG GAGAGACACG CATAGCAGAA
3660

AATGGACAAA AACTAACAAA TGAATGGGAA TTGTACTTGA TTAGCATTGA AGACCTTGTT
3720

TATACTATGA TAAATGTTTG TATTTGCTGG AAGTGCTACT GACGGTAAAC CCTTTTTGTT
3780

TAAATGTGTG CCCTAGTAGC TTGCAGTATG ATCTATTTTT TAAGTACTGT ACTTAGCTTA
3840

TTTAAAAATT TTATGTTTAA AATTGCATAG TGCTCTTTCA TTGAAGAAGT TTTGAGAGAG
3900

AGATAGAATT AAATTCACCT ATCTTACCAT CTAGAGAAAC CCAATGTAA AACTTTGTTG
3960

TCCATTATTT CTGTCTTTTA TTCAACATTT TTTTATAGAG GTGGGAGGAA TACAGAGGAG
4020

GTACAATGAT ACACAAATGA GAGCACTCTC CATGTATTGT TTTGTCCTGT TTTTCAGTTA
4080

ACAATATATT ATGAGCATAT TTCCATTTCA TTAAATATTC TTCCACAAAG TTATTTTGAT
4140

GGCTGTATAT CACCCTACTT TATGAATGTA CCATATTAAT TTATTTCTCTG GTGTGGGTTA
4200

TTTGATTTTA TAATCTTACC TTTAGAATAA TGAAACACCT GTGAAGCTTT AGAAAATACT
4260

GGTGCCTGGG TCTCAACTCC ACAGATTCTG ATTTAACTGG TCTGGGTTAC AGACTAGGCA
4320

TTGGGAATTC AAAAAGTTCC CCCAGTGATT CTAATGTGTA GCCAAGATCG GGAACCCCTG
4380

TAGACAGGGA TGATAGGAGG TGAGCCACTC TTAGCATCCA TCATTTAGTA TTAACATCAT
4440

FIGURE 28E

CATCTTGAGT TGCTAAGTGA ATGATGCACC TGACCCACTT TATAAAGACA CATGTGCAAA
4500

TAAAATTATT ATAGGACTTG GTTTATTAGG GCTTGTGCTC TAAGTTTCT ATGTTAAGCC
4560

ATACATCGCA TACTAAATAC TTTAAAATGT ACCTTATTGA CATACATATT AAGTGAAAAG
4620

TGTTTCTGAG CTAAACAATG ACAGCATAAT TATCAAGCAA TGATAATTTG AAATGAATTT
4680

ATTATTCTGC AACTTAGGGA CAAGTCATCT CTCTGAATTT TTTGTACTTT GAGAGTATTT
4740

GTTATATTTG CAAGATGAAG AGTCTGAATT GGTCAGACAA TGTCTTGTGT GCCTGGCATA
4800

TGATAGGCAT TTAATAGTTT TAAAGAATTA ATGTATTTAG ATGAATTGCA TACCAAATCT
4860

GCTGTCTTTT CTTTATGGCT TCATTAACCT AATTTGAGAG AAATTAATTA TTCTGCAACT
4920

TAGGGACAAG TCATGTCTTT GAATATTCCT TAGTTTGAGG AGAATATTTG TTATATTTGC
4980

AAAATAAAAT AAGTTTGCAA GTTTTTTTTT TCTGCCCAA AGAGCTCTGT GTCCTGAAC
5040

ATAAAATACA AATAACCGCT ATGCTGTTAA TTATTGGCAA ATGTCCCATT TTCAACCTAA
5100

GGAAATACCA TAAAGTAACA GATATACCA CAAAAGGTTA CTAGTTAACA GGCATTGCCT
5160

GAAAAGAGTA TAAAGAATT TCAGCATGAT TTTCCATATT GTGCTTCCAC CACTGCCAAT
5220

AACA
5224

51/57

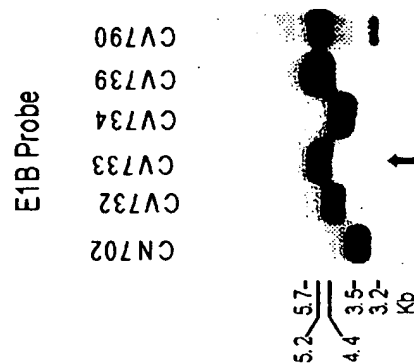


Figure 29B

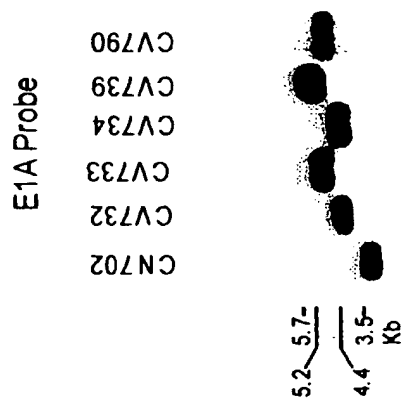


Figure 29A

52/57

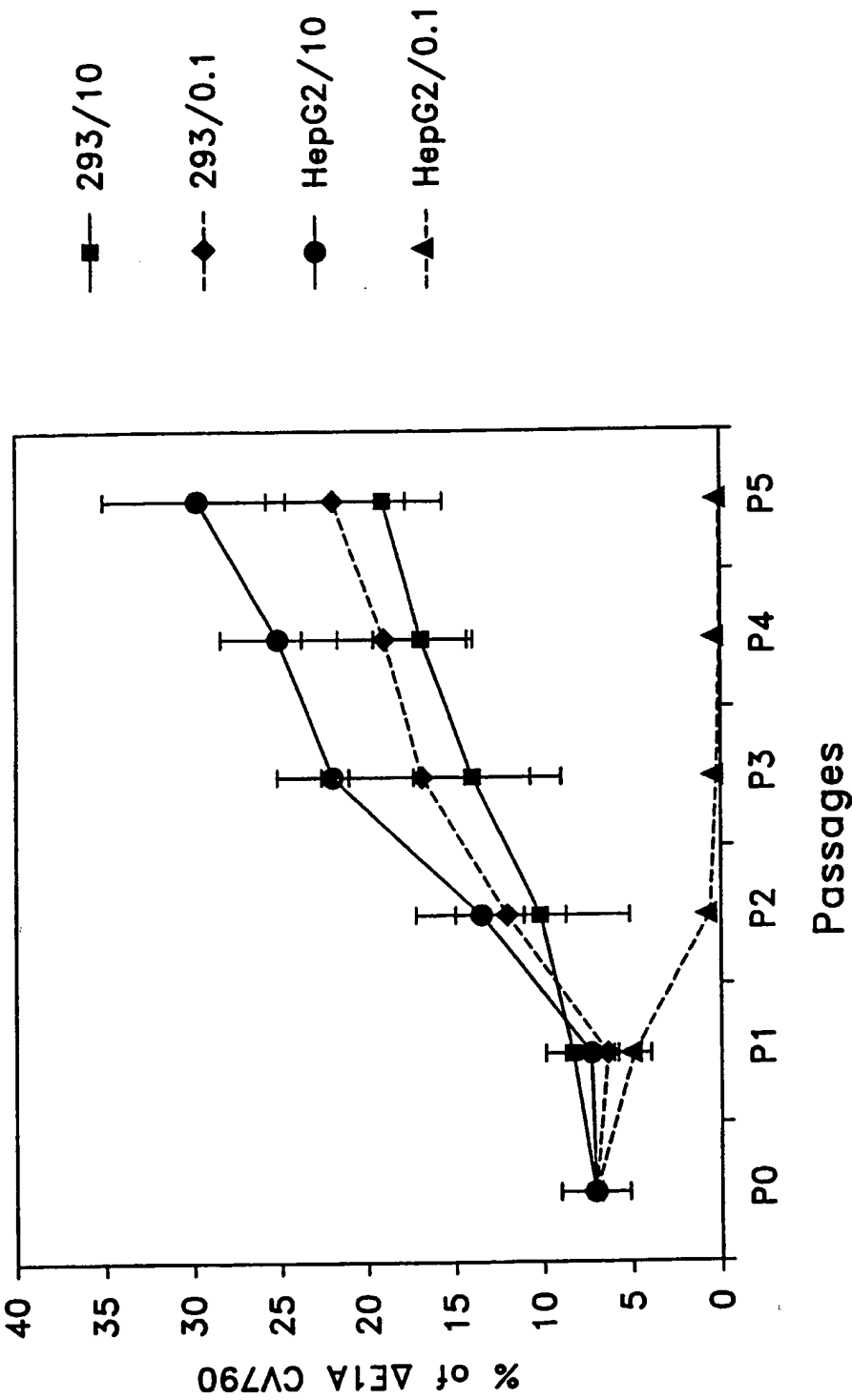
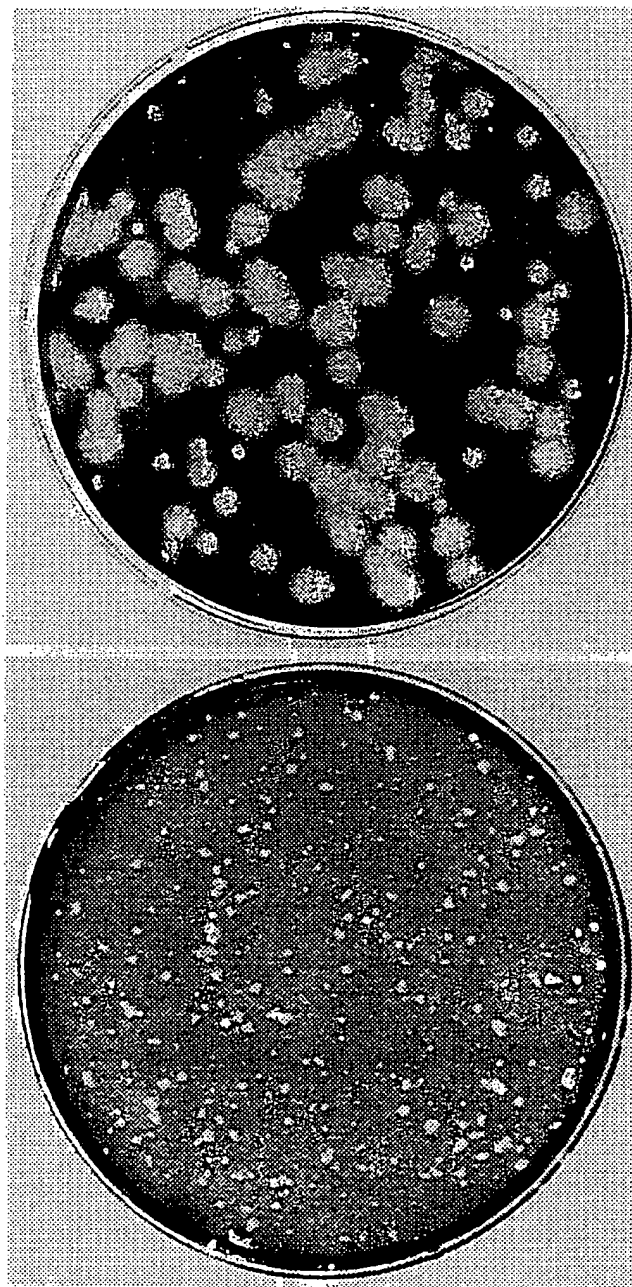


FIGURE 30

53/57

293 cells



CV790

CV733

Figure 31

54/57

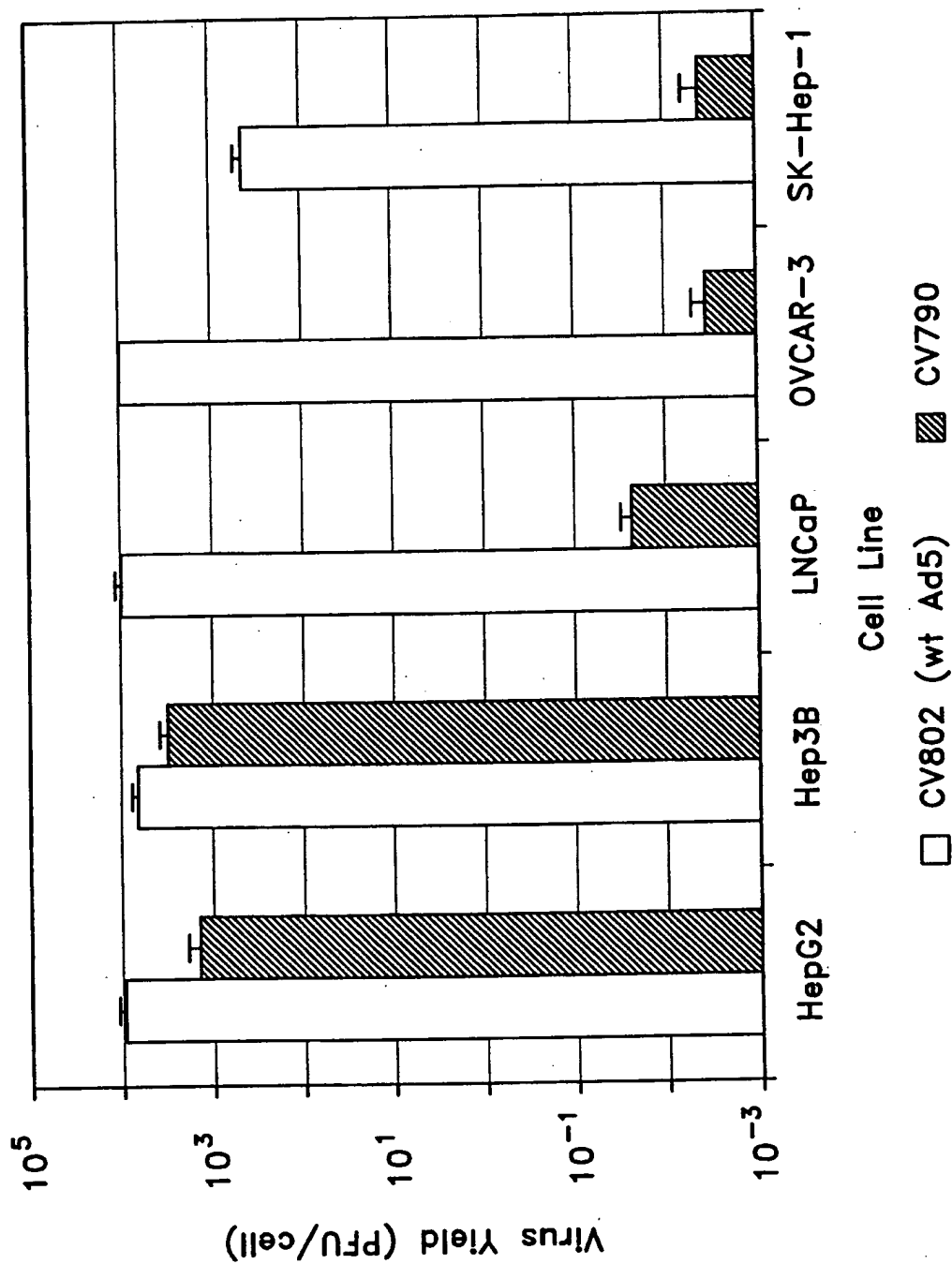


FIGURE 32

55 / 57

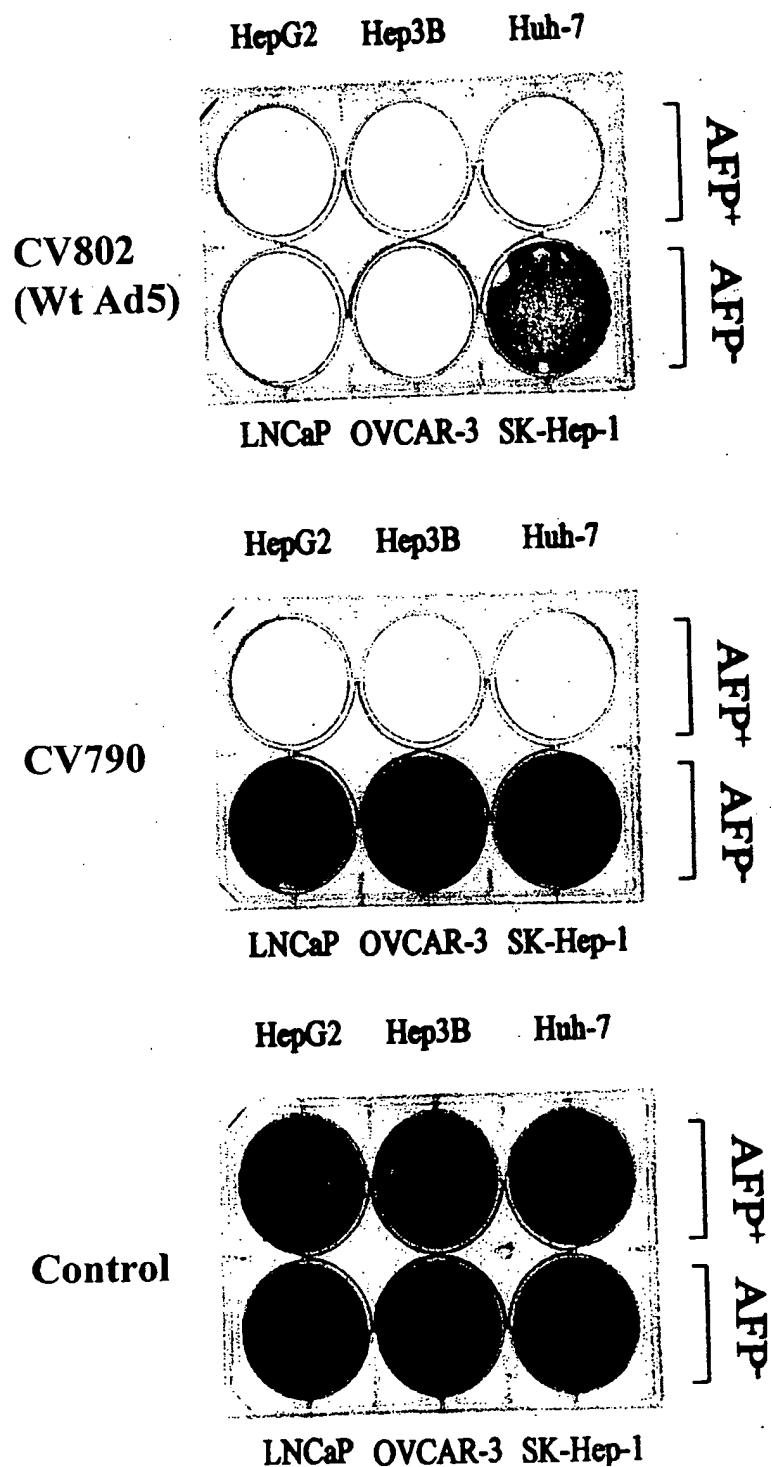


FIGURE 33

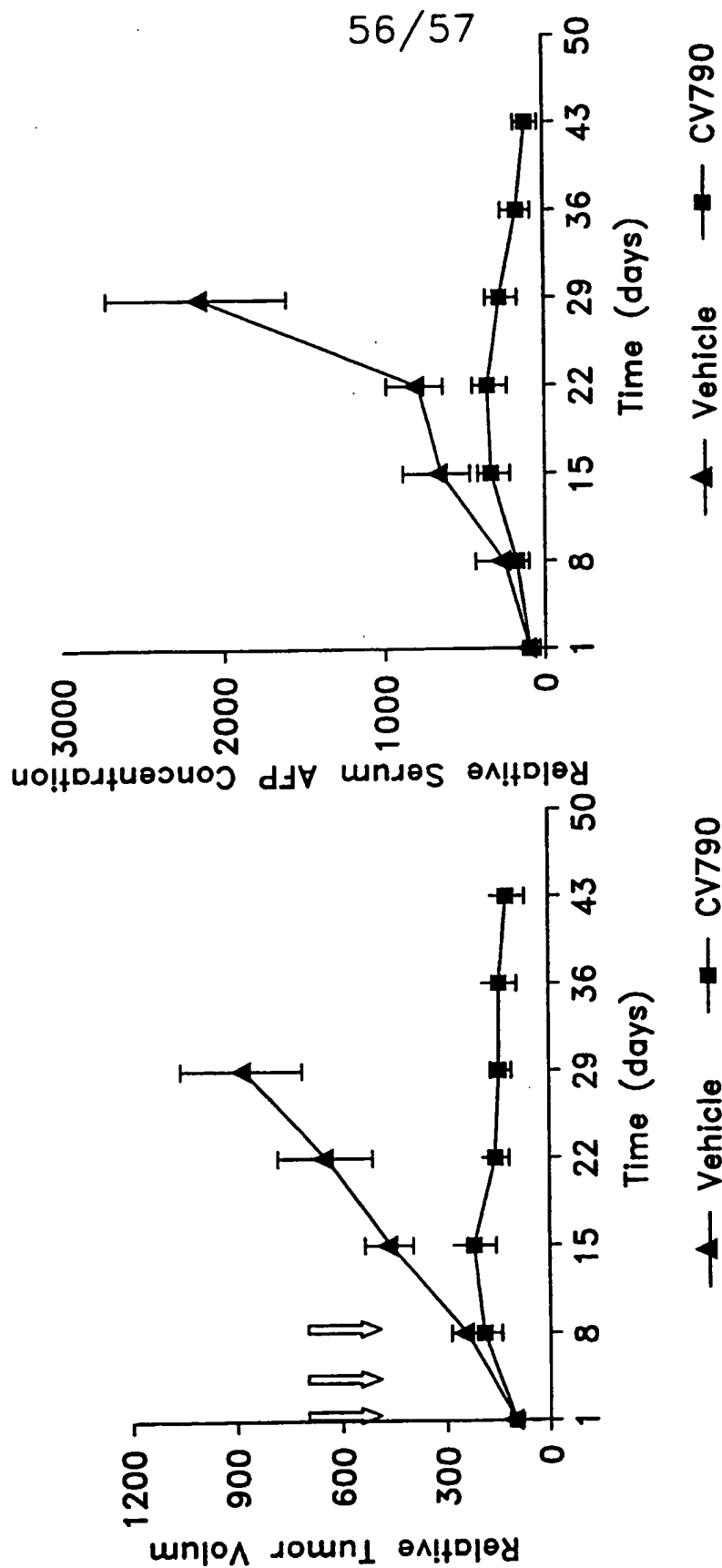


FIGURE 34B

FIGURE 34A

57/57

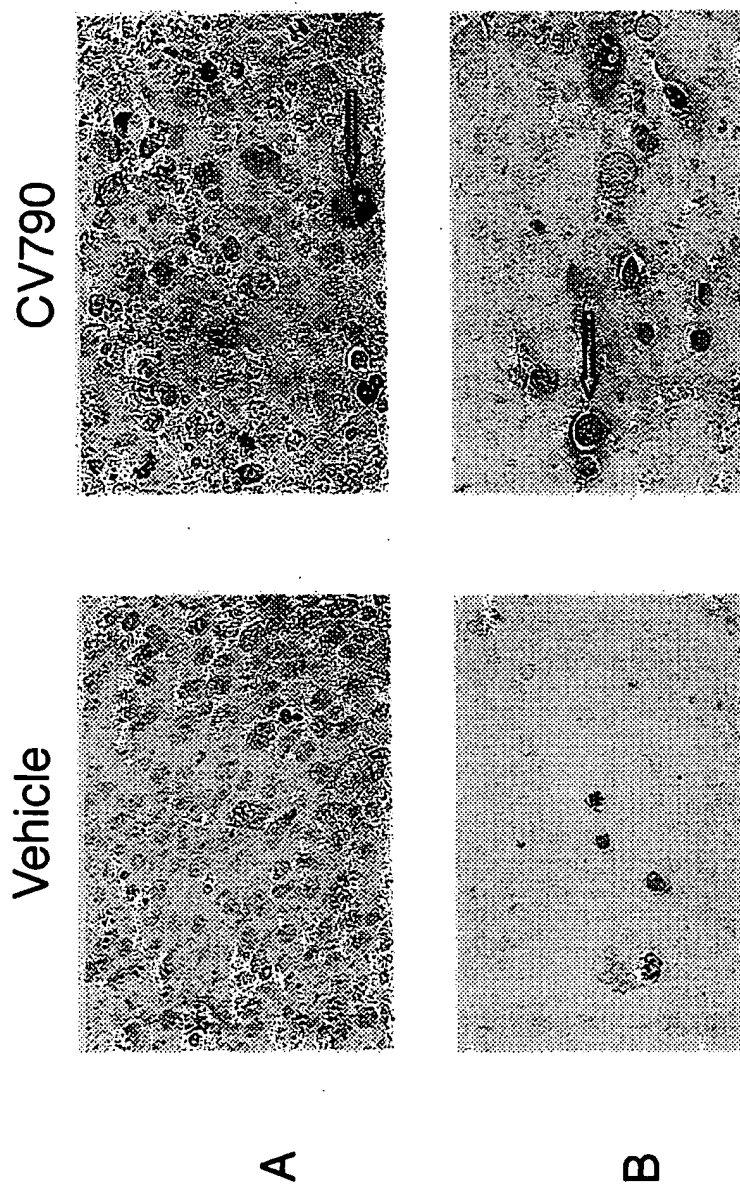


Figure 35